Accurate estimation of GFR in children using endogenous serum markers

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VRIJE UNIVERSITEIT

Accurate estimation of GFR in children using endogenous serum markers

Creatinine or cystatin C? - Both!

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor of Philosophy aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. V. Subramaniam,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
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door

Emil den Bakker

geboren te Jeruzalem, Israël

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copromotor: dr. A. Bökenkamp

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General introduction and outline of thesis

Glomerular filtration rate (GFR) is a key measure of kidney function.(1) It describes the rate by which fluids cross the glomerular filtration barrier, i.e. the total volume (in milliliters) of water that passes from renal capillaries into Bowman's capsule per minute. This rate depends on both renal and extra-renal factors such as the number of functioning glomeruli, the filtration surface regulated by the renal mesangium cells, the permeability of the basement membrane surrounding the renal capillaries, the hydrostatic pressure in Bowman's capsule, the colloid osmotic pressure of serum as well as the blood pressure in the afferent and efferent arterioles. (2, 3) GFR is positively correlated to blood volume, which is higher in larger individuals. However larger individuals also require a higher GFR to maintain homeostasis than smaller individuals. This is why GFR is often adjusted for body surface area [i.e. ml/min/1.73m^2], particularly in children. After glomerular filtration, the fluid collected in Bowman's capsule undergoes extensive tubular handling before it is voided as urine. This makes direct measurement of GFR exceedingly difficult as the rate of urine production can differ vastly from the rate of glomerular filtration. Therefore, in order to measure GFR exogenous markers are required that are inert, excreted exclusively through glomerular filtration and neither secreted nor reabsorbed by the renal tubules. The most accurate measurements involve injection of inulin, iohexol or ^{51}Cr-Technetium (4, 5) into the bloodstream and plotting the decline in serum concentrations by multiple serum measurements [called plasma clearance], (6-8) or by relating the amount of marker excreted in the urine to marker serum concentrations [called renal clearance] (5) or by determining the infusion rate necessary to attain a steady state of the marker in serum [called infusion clearance].(6) All these so-called gold standard methods for the measurement of GFR are invasive, costly and time consuming. Therefore in clinical practice GFR is generally estimated using serum levels of endogenous markers.

Endogenous markers of GFR

Like exogenous markers, endogenous markers should be excreted (almost) exclusively through glomerular filtration and have minimal tubular handling. As an additional requirement they should be synthesized at a steady rate.(9, 10) Several endogenous markers for kidney function have been characterized, the most commonly used in clinical practice being creatinine (11) and urea.(12) More recently a number of low-molecular weight protein markers have been studied: cystatin C, (13) beta-trace protein (BTP) (14) and beta-2 microglobulin (B2M).(15) This thesis will focus mainly on creatinine and cystatin C and to a lesser extent on beta-trace protein for the estimation of GFR.

eGFR equations

In order to translate serum levels of a marker into a corresponding eGFR, marker-specific equations are necessary. These equations differ depending on the population from which they are derived as well as the mathematical technique used to develop the equation. A more detailed introduction into both the mathematical tools and the different markers is given in chapter 1, which is a broad review article on this subject.

Outline of this thesis

The aim of this thesis is to improve GFR estimation using well-established and more recently recognized endogenous markers for kidney function. In order to achieve this goal it is imperative to know the molecular pathways and confounding factors of the individual markers.

In *section 1* the available markers are introduced, along with their confounding factors. **Chapter 1** is a review article providing an overview of the known markers. In this chapter known confounding factors are summarized for each marker and a comprehensive list of recent eGFR equations for children is provided.

One of the confounding factors described is the use of glucocorticosteroids, which impairs the accuracy of the low-molecular weight markers. **Chapter 2** addresses the question whether creatinine is similarly affected by steroid use.

For beta-trace protein (BTP) few eGFR equations have been established and less so for children. The existing equations were derived using linear regression of logarithmized data in mostly diseased populations, making confounding of the equation by patient-specific factors likely. In **chapter 3** we use a different approach by using normal values from a healthy population. Using this equation we compare accuracy and bias in different population subgroups, such as diagnosis, age, gender and steroid use.
In adults a group of patients has been identified in whom eGFR based on creatinine is consistently higher than eGFR based on cystatin C. It has been hypothesized that this phenomenon is due to altered size selectivity in the glomerular filtration barrier which affects the excretion of the larger cystatin C molecule more than creatinine and has been termed “Shrunken pore syndrome”. **Chapter 4** explores the existence of “Shrunken pore syndrome” in children, using the BTP-based equation from the previous chapter and its implications for GFR estimation.

Section 2 focusses on the combination of eGFR equations based on creatinine and cystatin C to improve accuracy.

Chapter 5 shows that the combination of a height-independent creatinine equation with a cystatin C equation improves accuracy of GFR prediction and allows for direct eGFR reporting by the laboratory without the need for anthropometric data.

Chapter 6 expands on the previous chapter, increasing the accuracy by comparing arithmetic and geometric means and using weighted means when there is a large difference between the two GFR estimations.
Section 3 comprises a general discussion and conclusions from this thesis.

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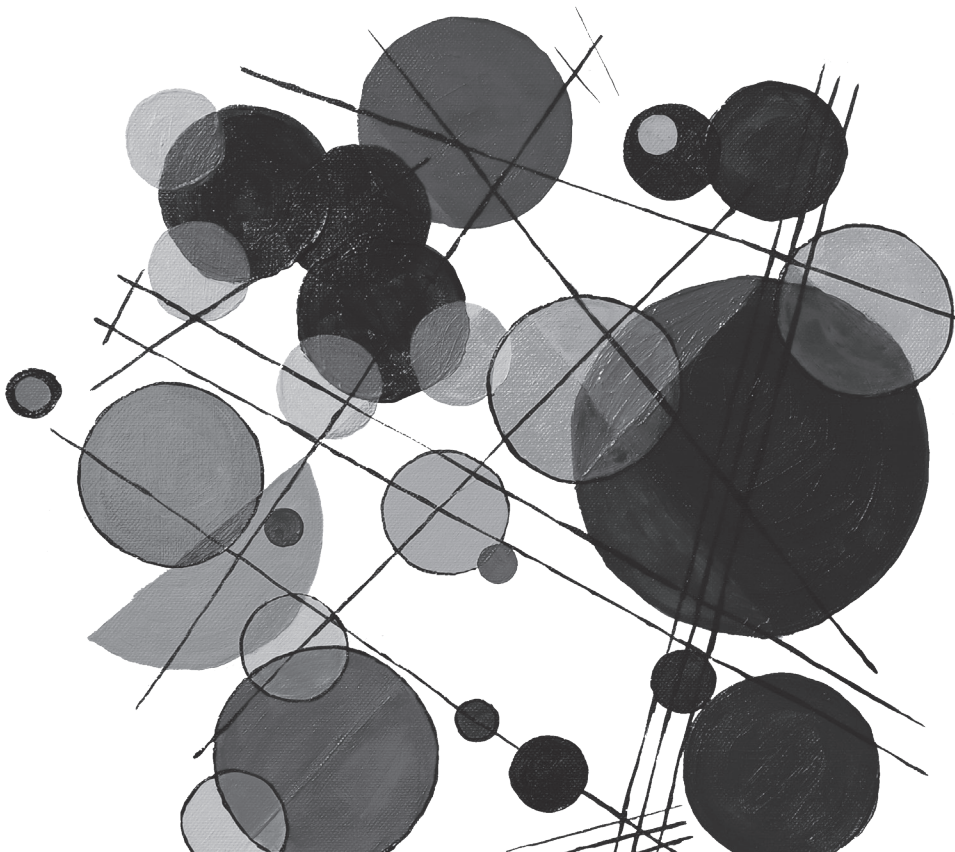
Section one

Exploring different markers



1

Endogenous markers for kidney function in children: a review

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Critical Reviews in Clinical Laboratory Science

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Abstract

Although glomerular filtration rate (GFR) in children can be measured using a gold-standard technique following injection of an exogenous marker, this invasive and cumbersome technique is not widely available and GFR is commonly estimated using serum levels of endogenous markers. Creatinine, urea, cystatin C, beta-trace protein and beta-2 microglobulin are well-established endogenous markers of kidney function. These markers differ in site of production, effects of diet and medication as well as renal-tubular handling and extra-renal elimination. For each marker, different methods are available for measurement. Importantly, the measurement of creatinine and cystatin C has recently been standardized with the introduction of international reference standards.

In order to allow estimation of GFR from serum marker concentrations, different estimating equations for GFR (eGFR) have been developed in children, using simple or more complex regression strategies with gold standard GFR measurements as dependent variable. As a rule, estimation strategies relying on more than one marker – either by calculating the average of single parameter equations or by using more complex equations incorporating several parameters - outperform eGFR estimations using only a single marker.

This in-depth review will discuss the physiology, measurement and clinical use of creatinine, urea, cystatin C, beta-trace protein and beta-2 microglobulin in children. It will also address the generation of eGFR equations in children and provide an overview of currently available eGFR equations for the pediatric age group.

Keywords
Cystatin C, creatinine, urea, beta-trace protein, beta-2 microglobulin, clearance study, estimated GFR, physiology

1. Introduction

Knowledge about glomerular filtration rate (GFR) in children is essential for the identification of renal disease, monitoring of the effect of interventions and disease progression as well as for adequate drug dosing and monitoring of drug toxicity.
GFR describes the rate of fluid passage across the glomerular membrane. As the rate of glomerular filtration strongly depends on the blood flow through the renal arteries, an increase in cardiac output with physical growth is associated with an increase in GFR. Therefore, absolute GFR (in ml/min) in adults is much higher than in children. To correct for differences in body size, GFR in children is normalized to the body surface area of an average adult, i.e. 1.73m^2 and expressed in ml/min/1.73m^2.

Besides renal blood flow the number of functioning nephrons is another important determinant of GFR. During fetal development, nephrons are formed until 32-36 weeks of gestation, (1) when the maximum number of nephrons has been formed. Although term neonates have the same number of nephrons as adults, their GFR is only around 20 ml/min/1.73m^2. (2) Maturation of the nephrons leads to a rapid rise in GFR in the first weeks of life. (3) Between the age of 1 and 2 years, GFR reaches adult levels (Figure 1).(4) Premature neonates, who are born before nephrogenesis has been completed, have lower renal mass at birth. In these children nephrogenesis continues for up to 40 days post-partum leading to an increase in renal function (5, 6), still extremely premature neonates may fail to achieve a normal number of nephrons.

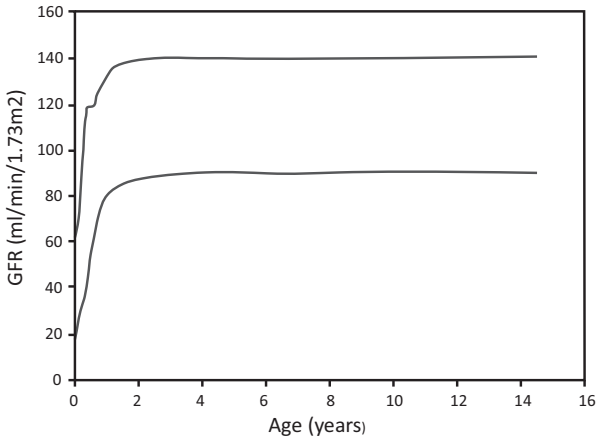

Figure 1: Development of GFR with age
Presentation of 5th and 95th centile of GFR measured by inulin clearance in healthy children. Data derived from Brodehl et al, 1982 (4)

As in adults, GFR in children can be measured by injecting an exogenous marker, which is inert and excreted exclusively via glomerular filtration. The golden standard technique for GFR measurement is the inulin clearance. During continuous infusion of inulin, a polyfructosan with a molecular weight of ca 5000 Da, the GFR is calculated from inulin serum concentrations and inulin excretion in timed urine collections.(7) This is a cumbersome procedure and in particular timed urine collections pose a special problem in children who may not be continent. As an alternative, plasma disappearance techniques following a single injection of one of several exogenous markers, i.e. inulin, (8) iohexol, (9) ^{51}Cr-EDTA, (10) ^{99m}Tc-DTPA (11) and iothalamate (12) can be used to measure GFR. Soveri et al (13) recently published a systematic review comparing the different techniques for measuring GFR and concluded that the plasma clearance of ^{51}Cr-EDTA, iohexol and inulin is sufficiently accurate to measure GFR, while ^{99m}Tc-DTPA and Iothalmate are only sufficiently accurate if performed as renal clearance with urine collection. While inulin is no longer available in many countries, iohexol, a radiocontrast agent administered at very low dose, is increasingly used. It can even be measured in capillary blood samples.(14, 15) In clinical practice, GFR is often measured by creatinine clearance by relating serum creatinine levels to timed urinary creatinine excretion. Even in adults, where incontinence is no major issue, endogenous creatinine clearance is insufficiently accurate (13) and should be abandoned. (16) Instead, estimated GFR (eGFR) based on serum concentrations of endogenous markers is recommended in international guidelines.(17, 18)

The scope of this review is to characterize endogenous markers of GFR currently available for clinical practice in pediatric populations. These markers will be explored for their biochemical and physiological characteristics relevant for the pediatric age group. Special emphasis will be given on how these markers can be used to estimate GFR in children.

2. Physiology of endogenous markers for kidney function

GFR can be estimated using endogenous serum markers. An ideal endogenous marker has the following properties:

1) Constant production rate
2) High glomerular sieving coefficient, i.e. free passage across the glomerular wall (19)
3) No protein binding
4) Excretion exclusively by glomerular filtration, i.e. no extra-renal metabolism and no renal tubular secretion or re-absorption of the intact molecule
5) Accurate measurement by automated assays at acceptable cost.

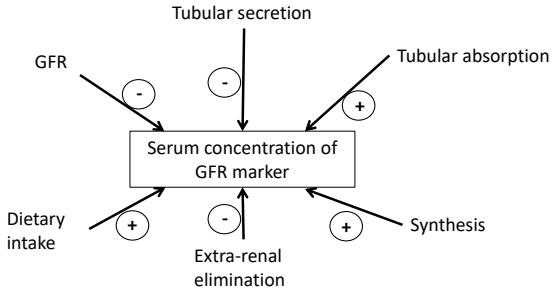

Figure 2: Factors influencing serum concentrations of endogenous eGFR markers

The serum concentration is determined by GFR, analytical variation, calibration of the assay as well as renal tubular and extra-renal mechanisms involved in accretion or elimination of the marker (Figure 2).

Several endogenous markers have been shown to meet many of the requirements for an endogenous GFR marker. Their characteristics are summarized in Table 1 and will be discussed in more detail below.

Table 1: Characteristics of endogenous marker for kidney function
GCS; glucocorticoid therapy

Marker	Molecular mass	Iso-electric point	Derived from	Volume of distribution	Interactions
Creatinine	113 Da	8.74	Muscle	Total body fluid	Muscle mass, cooked meat, fish, medication
Urea	60 Da		Protein catabolism	Total body fluid	Catabolic state, hydration, internal bleeding GCS
Beta-2 microglobulin	11 800 Da	5.4-5.7	All nucleated cells	Extra-cellular fluid	Viral infections, malignancies, GCS
Cystatin C	13 300 Da	9.30	All nucleated cells	Extra-cellular fluid	Thyroid dysfunction, GCS
Beta-trace protein	23 000 Da	5.8-6.7	Cerebrospinal fluid	Extra-cellular fluid	GCS

2.1. Creatinine

2.1.1 Physiology

Creatinine is the most commonly used marker in children and adults alike. Creatinine originates from the creatine/phosphocreatine pathway. Creatine is synthesized in the kidneys and the liver (20) and stored mainly in striated muscle cells, (21, 22) where it is phosphorylated to phosphocreatine by creatine kinase. In turn, phosphocreatine is used to phosphorylate ADP into ATP when energy demand is high. Both creatine and phosphocreatine spontaneously degrade to creatinine. Besides endogenous creatinine production, dietary intake of cooked meat and fish may contribute to the creatinine pool and affect serum creatinine levels. This also applies to creatine supplements. (23, 24)

Creatinine is a small molecule with a molecular weight of 113 Da and an iso-electric point of 8.74. Creatinine is freely filtered across the glomerular membrane making glomerular filtration the principal route of elimination. However, creatinine is also excreted by tubular secretion, the level of which is inversely related to GFR. (25, 26) Drugs known to inhibit tubular creatinine secretion are trimetoprim, cimetidine and fenofibrate. Their use may lead to higher creatinine concentrations, which do not indicate a deterioration of glomerular filtration. As a result of tubular creatinine secretion, the rise in serum creatinine may be blunted until GFR has almost halved, a phenomenon denoted as “creatinine-blind range”. This is most prominent in children who have low muscle mass and physiologically low serum creatinine levels (Figure 3). Conversely, if urine leaks into the abdomen or the perirenal space, creatinine will be re-absorbed leading to falsely elevated serum concentrations.(27) In patients with severe kidney failure gut creatininase also contributes to creatinine excretion (28), which can be inhibited by antibiotic therapy leading to a rise in serum creatinine.(18)

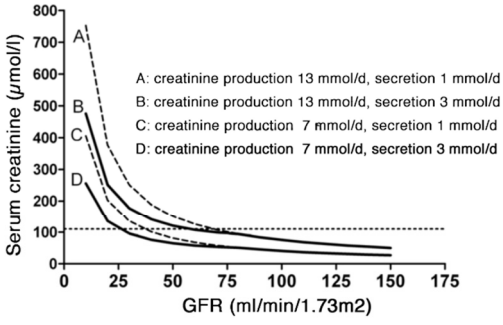

Figure 3: Serum creatinine versus GFR for different rates of creatinine production and tubular secretion
By courtesy of Professor Jack F.M. Wetzels, Nijmegen (modified)

The volume of distribution of creatinine is total body water.(29) Therefore, creatinine serum concentrations lag behind acute changes in GFR. This is most marked at low GFR when it may take several days until a new steady-state has been reached (Figure 4).(30)

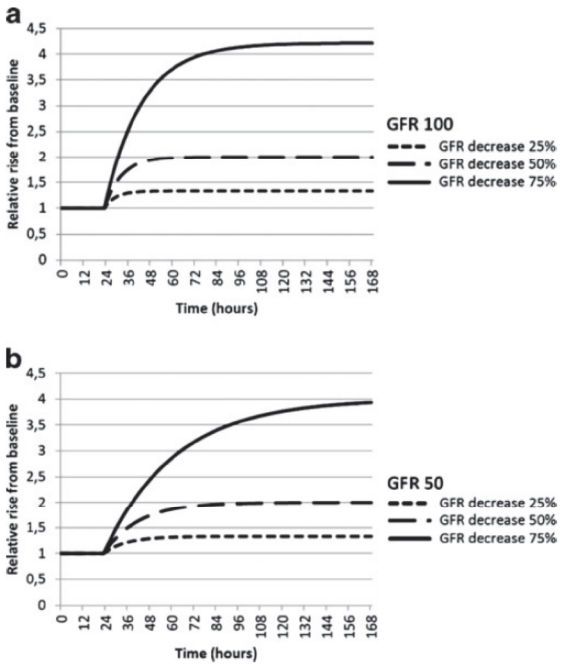

Figure 4: Model of changes in serum creatinine
Acute decrease in GFR by 25, 50 or 75% at 24 hours. a; Baseline GFR 100 ml/min/1.73m^2, b; baseline GFR 50 ml/min/1.73m^2. From Slort et al, 2012 (30), used with permission

2.1.2 Analytical methods

There are several methods of measuring creatinine. The most common and least expensive Jaffe method uses alkaline picrate, which changes to a red color in the presence of creatinine. (31) This method is hampered by so-called non-creatinine chromogens, which are most relevant at the very low creatinine concentrations typically found in infants.

This problem is overcome when using enzymatic creatinine assays. (32-34) Although comparative studies have shown that the enzymatic methods have less interference, the Jaffe method is still widely used, due to its low cost. (35-37)

Neonates in the first week of life have physiologically high serum bilirubin levels due to hemolysis of fetal erythrocytes, underdeveloped hepatic conjugating capacity and an increased enterohepatic cycle. (38) Bilirubin absorbs light in roughly the same spectrum as the chromogens formed in the Jaffe reaction. In the alkaline milieu of the Jaffe reaction bilirubin is oxidized to biliverdin causing a decrease in absorbance at the wavelength of 520 nm used to measure creatinine, while the creatinine-picrate chromogens cause an increase. This leads to underestimation of creatinine concentrations in patients with high bilirubin levels. (39) This is even more so for premature infants. Fortunately enzymatic assays are far less subject to this interference. (40) Therefore, the use of enzymatic tests for creatinine is mandatory in the neonatal period and should be used preferably in all children due to lower muscle mass.
The SI unit of creatinine is µmol/l while in many parts of the world creatinine is reported in mg/dL (conversion SI x 0.0113 = mg/dL).

2.1.3 Reference values in children

Until the widespread implementation of isotope dilution mass spectroscopie (IDMS)-based calibration of creatinine measurement, reference values varied between hospitals. The use of the IDMS-based standard has allowed establishing uniform reference ranges over the whole age spectrum. (41) Normal values for serum creatinine levels are highly age-dependent. Neonates have relatively high serum creatinine directly post-partum, reflecting maternal levels due to diaplacental exchange of creatinine. (42) Serum creatinine then drops reflecting low endogenous production in infancy, with the lowest normal values found at about 2 months of age. From then on serum creatinine levels rise steadily as a result of increasing muscle mass (Figure 5).(43) (44) Until puberty there is no clear gender-specific difference, while from the age of 14 normal values in male adolescents are higher than in females. (41) As muscle mass in children is more closely linked to height as opposed to weight or body surface area, (45) eGFR equations based on creatinine use height as a correcting factor.

Unless detailed reference intervals per year of life are used, conversion of measured creatinine concentrations into a creatinine-based eGFR is mandatory for the recognition of impaired renal function in children. (17)

2.1.4 Considerations regarding the use of creatinine in children.

A major problem with estimating kidney function in the neonatal period is the diaplacental exchange of creatinine between mother and fetus. Being a small molecule creatinine passes the placental wall freely and there is a high correlation between maternal and neonatal serum levels.(42) This precludes kidney function assessment using creatinine

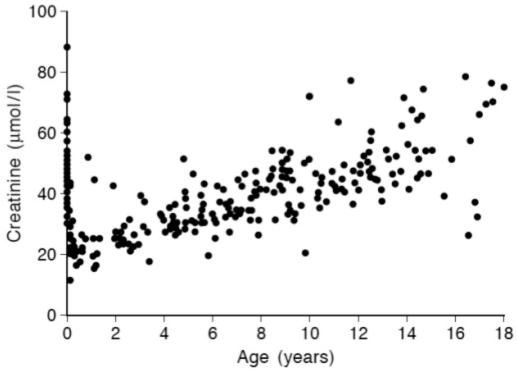

Figure 5: Serum creatinine versus age
Data from Bökenkamp et al (43), used with permission.

both in utero (using cord blood) (46) and directly post-partum, when serum creatinine reflects the kidney function of the mother rather than the newborn. Creatinine is therefore a poor marker for acute kidney injury from perinatal asphyxia.(47) It may take serial measurements during the first days of life to determine if the kidney function of a neonate is normal.

As in adults, muscle mass largely determines serum creatinine concentrations. This is most relevant for boys during adolescence. As the start of puberty varies by up to 5 years, purely age-related reference intervals may be misleading in patients with very early or late puberty. This may affect the early recognition of acute renal failure using the pediatric RIFLE (risk, injury, failure, loss and end-stage renal failure) criteria.(48) As the rise from a baseline creatinine concentration is one of the diagnostic criteria, early stages of acute kidney injury are easily missed if this crucial information is not available.

Other populations at risk when using creatinine are children with anorexia nervosa, malignancy, advanced liver disease or neuromuscular disease (e.g. muscle dystrophy, spina bifida).(49) Also in children after liver transplantation, GFR is overestimated when using creatinine.(50, 51) This also applies to young children after transplantation of a kidney from an adult donor. These children have extremely low serum creatinine concentrations potentially leading to a delayed recognition of allograft dysfunction.(52) In order not to miss early signs of kidney dysfunction in particular in young children, it is imperative to use the enzymatic creatinine assay and when reporting concentrations in mg/dL, to report with two instead of one digit, the latter being common practice in many laboratories.

As the volume of distribution of creatinine is the intra- and extracellular space, there is a considerable time lag until establishment of a new steady state after acute changes in kidney function (Figure 4).(30) This is most marked in newborns in which total body water may be up to 75% of body weight as opposed to older children (around 60% of total body weight).(53)

2.2. Urea

2.2.1 Physiology

Urea is formed in the urea cycle, a series of enzymatic steps to neutralize ammonia, which is released with degradation of amino acids.(54) It is a small nitrogen containing compound, with a molecular weight of 60 Da. Due to its small size it passes the glomerular barrier freely, making the concentration of urea in the initial filtrate virtually identical to that of serum, which is one of the prerequisites of a GFR marker. However, the walls of some segments of the renal tubules are also permeable to urea resulting in complex tubular handling.(55) Urea reabsorption is by a facilitated passive process through urea transporters on the apical membrane. Expression of the urea transporters in the thin descending limb of Henle and the medullary collecting duct is increased by ADH leading to increased urea reabsorption in anti-diuresis. Therefore, urea clearance is directly related to urine flow and varies by some 300% between anti-diuresis and maximal urine dilution.(56) Other extra renal factors affecting its serum levels include dietary protein intake (+), internal bleeding (+), glucocorticosteroid treatment (+), catabolic state (+) and intestinal excretion (-).(57, 58)

Urea is found both intra- and extracellularly, its volume of distribution is the total body water.

2.2.2 Analytical methods

Urea can measured using a direct colorization or an enzymatic method. In the Fearon method urea gives a bright yellow color after addition of diacetyl monoxime, which turns orange after oxidation.(59, 60) Enzymatic methods use urease to break down urea into ammonium, which in turn is measured by the rate of decline of NADH.(61)
The SI unit of urea is mmol/l while in many parts of the world urea is reported in mg/dL (conversion SI x 60.06 = mg/dl). Others report blood urea nitrogen (BUN) in mg/dL where only the nitrogen content of urea is given (conversion SI x 2.8 = mg/dL).

2.2.3 Reference values in children

Several papers containing age specific reference values of serum urea have been published. (62, 63) Unlike creatinine, serum urea levels are not clearly age-related but rather reflect fluid and protein intake and metabolism. Urea concentrations are slightly lower in growing children compared to adults, in particular in newborns.(64)

2.2.4 Considerations regarding the use in children.

As with creatinine, serum levels directly post-partum are closely related to maternal values due to diaplacental exchange. Also, since the fetus depends on diaplacental urea exchange for growth, serum levels directly after birth are more indicative of the neonate's metabolic rate than its kidney function.(65) As described above, volume depletion increases renal tubular uptake, causing an exaggerated rise in serum urea.(66) This can be used for the distinction between renal and pre-renal acute renal failure.(67) Volume depletion due to diminished intake or gastrointestinal losses is far more prevalent in children than in adults. (68) This makes urea even less suitable as a marker of GFR in pediatric populations.

2.3. Cystatin C

2.3.1 Physiology

Cystatin C is a low molecular weight protein of 13.3 kDa with an iso-electric point of 9.2. It is part of the cystatin family of anti-proteinases. In the extracellular compartment, cystatin C forms tight, reversible bonds with cysteine proteinases, thereby neutralizing their proteolytic activity.(69, 70) In the past, cystatin C was also denoted as γ-CSF, γ-trace, post-γ-protein or post-γ-globulin (71) until it became clear that its properties placed it in the cystatin family.(72)

Cystatin C is produced by nearly all nucleated cells (73) and has been found in all body fluids. This explains the clinical observation by Andersen et al (74) that inclusion of body cell mass improves the predictive performance of an eGFR equation. The highest concentrations were found in cerebrospinal fluid.(75) Unlike other members of the cystatin family, cystatin C is not found intracellularly. Therefore, its volume of distribution is limited to the extracellular space. This leads to a higher sensitivity for changes in GFR compared to creatinine whose volume of distribution is total body water.(30) In line with this, serial measurements showed higher *intra*-individual variability of cystatin C compared to creatinine, where a higher *inter*-individual variability was found.(76) This results in higher sensitivity of cystatin C compared to creatinine for the early recognition of acute kidney injury reported by a large number, (77-80) but not all studies on this subject.(30, 81, 82)

Cystatin C is not protein-bound. Due to its small size and positive charge it passes the glomerular membran easily with a sieving coefficient of 0.84.(83)

Tenstad et al reported that the renal extraction rate of radiolabeled cystatin C in rats was 94% of the ^{51}Cr-EDTA clearance.(84) Like other low-molecular weight proteins (85) filtered cystatin C is reabsorbed in the proximal tubule by megalin-cubulin receptor-mediated endocytosis and catabolized intracellularly.(84) Therefore, concentrations in the urine are very low (84) unless there is significant tubulointerstitial damage.(86, 87) Nephrotic range proteinuria leads to saturation of the re-absorptive capacity for cystatin C (and other low-molecular weight proteins) resulting in spilling of these proteins, which disappears when the nephrotic syndrome is in remission. (88)

Therefore, cystatin C is only suitable as a marker of GFR when measured in serum and it is not possible to calculate a “cystatin C clearance” in analogy to the creatinine clearance by using timed urine collection.

Being encoded by a house-keeping gene, cystatin C synthesis is not regulated, (89) its mean production rate is 0.117 mg/min/1.73m^2.(90) Still, glucocorticosteroids have been shown to increase cystatin C concentrations in a dose-dependent manner(91-93) due to induction of the promotor of the cystatin C gene.(94) Hyperthyroidism leads to higher cystatin C levels, while the opposite is true for the hypothyroid state. (95) These changes disappear after normalization of thyroid hormone concentration and have little effect on the performance of cystatin C for the diagnosis of acute kidney injury. (30, 96, 97) Epidemiological studies in adults showed a correlation between cystatin C levels and obesity and smoking. (98) Although cystatin C is no acute phase reactant as demonstrated in patients undergoing surgery (99) and during febrile illness, (100) serum concentrations are correlated with C-reactive protein, (101) a well-established marker of micro-inflammation. As cystatin C is a predictor of cardiovascular and all-cause mortality *independent* of kidney function (102) micro-inflammation may be the common denominator of these findings, (101) possibly as the result of impaired renal filtration of cytokines with comparable size of cystatin C. (103)
Although elimination of cystatin C is largely through glomerular filtration, there is also a constant extrarenal clearance of 22 ml/min/1.73m^2, (84, 90) which accounts for the observation that cystatin C concentrations do not exceed 7 mg/l even in anephric patients. (104)

Ca. 10% of Swedish patients harbor a polymorphism in the cystatin C promotor leading to ca. 0.05 mg/l lower cystatin C serum concentrations.(105) In US children, median cystatin

C in non-Hispanic blacks is 0.045 mg/l and in Mexican Americans 0.02 mg/l lower than in a white reference population.(106)

2.3.2 Analytical method

There are several methods for measuring serum concentrations of cystatin C. Particle enhanced immunoassays using beads coated with anti-cystatin C antibodies for turbidometric (PETIA) or nephelometric (PENIA) measurement (107, 108) or an immunofluorescence-based assay.(109) Some data suggest that the PENIA and the immunofluorescence assay are more accurate than the PETIA assays.(110-112)

Until 2010, no standardized calibrator was available for the different cystatin C assays. This precluded direct comparison of cystatin C measurements performed by different assays. (113) This problem has been solved by the generation of the IFCC-certified calibrator ERM-DA471, which is now used by all manufacturers of cystatin C assays. (114) A recent initiative to optimize the performance of 6 commercially available PETIA and PENIA assays has greatly improved the variability between these assays and helped to further standardize cystatin C measurement.(115)

For different cystatin C assays interference of bilirubin, lipids and hemoglobin at normal levels are not problematic,(116) while Akbas et al reported interference of hyperlipidemia exceeding 1000 mg/dL on a Siemens nephelometer.(117) Therefore an equation has been developed to correct measured cystatin C levels for the effect of triglycerides.(118)

2.3.3 Reference values in children

Although cystatin C does not cross the placental barrier (46) the highest serum concentrations of cystatin C are found in the neonatal period and slowly decrease during the first year of life (Figure 6).(43) This reflects the maturation of GFR in this period (Figure 1). After the age of one year, cystatin C concentrations stabilize and are similar to those in adults.(43, 119) While most studies found no gender-differences with cystatin C levels (43, 112), Groesbeck et al observed 0.092 mg/l higher cystatin C concentrations in females. They also noted higher cystatin C levels around the peak growth spurt (i.e. 12 years in girls, 14 years in boys). (106)

As the calibration of cystatin C assays has changed with time (120) only reference ranges established using the IFCC calibrator (114) are applicable nowadays. IFCC calibrated reference values for the first year of life in premature infants have been published by Nakashima et al., ranging from a mean of 1.776 mg/l in the first month to 0.9660 mg/l between 12 and 14 months.(121) They found no correlation between gestational age and

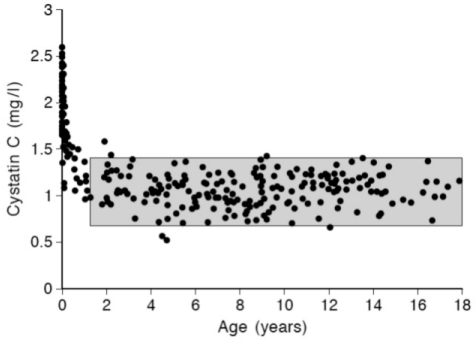

Figure 6: Serum cystatin C versus age
Grey area indicates reference range (DAKO PETIA, calibration 1998) First published by Data from Bökenkamp et al (43), used with permission.

serum concentrations, suggesting the data might be extrapolated to term neonates as well. A recent study among healthy children aged 4-17 years in Greece (122) showed mean cystatin C levels of 0.79 mg/l, which is comparable with Groesbeck's data of 0.76 mg/l before puberty and 0.82 mg/l in adolescents.(106)

2.3.4 Considerations regarding the use in children.

Unlike for creatinine and urea, there is no correlation between maternal and neonatal serum cystatin C concentrations suggesting that there is little to no transplacental exchange of cystatin C. (123) Therefore, cystatin C concentrations in cord blood can be used to assess kidney function of the fetus (46) and the newborn. It may be particularly useful for the diagnosis of acute kidney injury following perinatal asphyxia.(47)
Like the other markers, cystatin C can be measured in very small sample volumes, which is particularly important for small children. As venipuncture may be challenging in small children, cystatin C measurement in capillary samples has been explored. Although promising results were reported in adults, (124) this could not be reproduced in children where the capillary samples yielded lower cystatin C concentrations than paired venous samples.(125)

As noted earlier, serum creatinine is a poor predictor of acute kidney injury in children. Although there are little data on children using cystatin C in this setting, (80) it may be a more sensitive marker due to its smaller volume of distribution and the constant reference range in children older than 2 years of age .

Cystatin C has been shown to be a more accurate marker than creatinine in specific pediatric populations, such as children with active malignancy,(126) neural tube defects (127) and post liver transplant.(128) This can be explained since these are all populations in which muscle mass is diminished.

2.4. Beta-trace protein.

2.4.1 Physiology

Beta-trace protein (BTP), also known as Lipocalin-type prostaglandin D synthase, is a small glycoprotein of 190 aminoacids with a molecular weight ranging from 20 to 31 kDa depending on the degree of N-glycosylation.(129)

BTP is synthesized mainly in the central nervous system by glial cells and the choroid plexus and forms one of the principal constituents of the cerebrospinal fluid. (130) It catalyzes the reaction from prostaglandin H2 to prostaglandin D2, which plays a central role in nociception, temperature and sleep regulation. It is also involved in the transport of lipophilic molecules such as bilirubin, thyroid hormones, retinoid and amyloid-beta. (131-133)

The highest concentrations of BTP are observed in cerebrospinal fluid, making it a marker for distinguishing between cerebrospinal fluid leak and other bodily fluids.(134) Decreased cerebrospinal fluid concentrations were reported in bacterial meningitis, while concentrations in viral meningoencephalitis were normal. (135) Serum BTP originates from resorption of cerebrospinal fluid.(136) Subsequently, the liver eliminates BTP molecules with smaller carbohydrate residues, reducing the molecular weight range of BTP in serum to 26-29 kDa.(137) Additionally there is evidence for BTP originating from kidneys, genital organs and the heart, as summarized in an extensive review by Filler et al.(138) Although being the largest of the three low-molecular weight protein GFR markers, BTP is eliminated almost exclusively via the kidneys (139) and serum BTP concentrations are closely linked to GFR.(130, 140-142)

While urinary excretion of all low-molecular weight proteins increases with declining GFR due to impaired tubular reabsorption as mentioned earlier, urinary BTP outperforms the other markers as an early marker of impaired kidney function. In a study by Donadio et al, urinary excretion of BTP was inversely related to GFR and increased already when GFR fell below 90 ml/min/1.73m^2. (143) The authors hypothesize that the reabsorption capacity for BTP is lower than for the other markers. As loss of nephrons leads to increased BTP filtration in the remaining nephrons, due to increased serum levels and hyperfiltration,

this causes overflow at the tubular level.(144) Of note, this is a different mechanism than the concept underlying inulin or creatinine clearance using timed urine and simultaneous plasma sampling.

Glucocorticoids have been shown to decrease serum BTP concentrations in a dose-dependent manner.(145) The presence of a certain single nuclear polymorphism upstream of the BTP gene locus has been associated with 5% higher BTP concentrations.(146)

Due to its large molecular size, BTP does not cross conventional hemodialysis membranes. This makes it a useful marker of residual renal function in dialysis patients as opposed to urea and creatinine, which are eliminated by dialysis.(147) Due to the larger pore size BTP crosses high-flux or super high-flux membranes, and is less suited as a marker of residual renal function in these dialysis modalities.(148)

2.4.2 Analytical methods

The most commonly used method for BTP measurement is by particle-enhanced nephelometry as described previously for cystatin C. Alternative methods are ELISA and immunofluorescence.(137) It should be noted that there is no international calibrator for BTP measurement, which hampers comparison of measurement performed with different assays.

2.4.3 Reference values in children

As with the other markers BTP serum levels are highest directly after birth in both term and preterm infants.(149) These levels drop markedly in the first days after birth and more gradually in the first two years. From two years of age reference values using the Siemens PENIA assay stabilize around 0.7 mg/l (upper limit 1.0 mg/l) and are not affected by age or sex.(130, 140) However, a trend towards decreasing values with age is found and in adult populations lower mean values of 0.56 mg/l have been described.(150)

2.4.4 Considerations regarding the use in children.

Although not formally tested, it is very likely that the findings showing absence of diaplacental exchange of cystatin C and beta-2 microglobulin (46) can be extrapolated to BTP. This is supported by a study in which BTP concentrations in venous and arterial umbilical cord blood were identical, suggesting neither diaplacental exchange nor placental synthesis or degradation of BTP.(151) Also with regard to general pediatric patient populations, BTP appears to perform comparably to cystatin C and beta-2 microglobulin.(140) In pediatric patients with neural tube defects BTP is more closely correlated to GFR than creatinine, but is outperformed by cystatin C.(127) Little is known

about the accuracy of BTP in other specific pediatric populations such as malignancy and transplanted patients.

2.5. Beta-2-microglobulin

2.5.1 Physiology

Beta-2-microglobulin (B2M) is a small protein with a molecular weight of 11.8 kDa.(152) Being the beta chain of the major histocompatibility complex (MHC), it is found on the surface membrane of nearly all nucleated cells. (153) It is shed during membrane turnover and can be detected in various body fluids, most notably in serum and synovial fluid.(154, 155) Unbound B2M passes the glomerular wall with a high sieving coefficient. (156, 157) Like other low-molecular weight proteins, it is reabsorbed and catabolized in the proximal tubules.(158) Therefore elevated concentrations of B2M in urine are indicative of tubular dysfunction.(159)

Originating from the MHC, serum B2M concentrations not only reflect renal function but are also linked to viral infections (160, 161), inflammation (155) and various types of malignancy,(162, 163) where elevated levels have been reported. The ratio of cystatin C and B2M in simultaneous blood samples has been proposed as a diagnostic parameter for post-transplant lymphoproliferative disease.(164) Glucocorticosteroids decrease B2M concentrations in a dose dependent manner.(93) These extrarenal factors impair the usefulness of B2M as a marker of GFR.

Unlike the other low-molecular weight protein markers of GFR, which are considered non-toxic, severe chronic elevation of B2M in dialysis patients leads to the development of amyloidosis. (165) Therefore, modern dialysis techniques aim at removing not only small solutes but also larger molecules like B2M.

2.5.2 Analytical methods

Like the other LMW protein markers, B2M can be measured in serum using particle enhanced nephelometry or turbidometry. (166, 167) Alternatively, ELISA assays can be used.(168) As for BTP, there is no international calibrator, which hampers comparison of measurements performed with different assays.

2.5.3 Reference values in children

For B2M too, the highest serum concentrations are found in early infancy and decrease in the first two years of life. Several authors report a constant reference range of 1.19 to 2.25 mg/l for both genders from 2 years of age, (140, 169) while others found decreasing

concentrations with a slope of -0.034mg/l/year between the ages of 2 and 18 years.(170) The global reference range in this publication was comparable (i.e. 1.0 to 2.3 mg/l) to the other reports, however.

2.5.4 Considerations regarding the use in children

Like cystatin C, B2M does not cross the placental barrier and can therefore be used as a parameter of fetal renal function. (46) Several authors have demonstrated that B2M measured in cord blood can be used to predict poor kidney function in fetuses with severe bilateral hydronephrosis.(171, 172)
Although serum B2M has been used as a tool for evaluation of kidney function in pediatric populations with malignancy in research settings,(173) it is unclear whether there are added benefits of B2M compared to other markers in these specific pediatric populations.

3. Estimation of GFR from endogenous marker concentrations

3.1 Considerations regarding the development of eGFR equations

While marker concentrations refer to upper limits of the reference range to distinguish between normal and diminished kidney function, the concept of estimated GFR (eGFR) aims at further quantifying the degree of renal dysfunction and classification of chronic kidney disease (CKD) using the CKD-staging system.(18) It also provides a quantitative measure of changes in kidney function and can be used for dose calculation of drugs eliminated by the kidneys.

These equations are developed using the correlation between measured GFR (mGFR) based on a gold standard technique and simultaneous marker concentrations, anthropometric data and potential other covariates reflecting the extra-renal factors on marker concentration like gender, underlying diagnosis or the use of specific medication (Figure 7). While a number of confounders are known and can potentially be corrected for, other factors are unknown and introduce bias and variability depending on the population studied. Therefore, any given eGFR equation reflects the analytical method and the population used for its generation, which may not be applicable to other populations (and most importantly) to a specific patient. (174, 175) This underscores the need for thorough external validation and also explains the large number of eGFR equations published in the last three decades, starting off with the Modification of Diet in Renal Disease (MDRD) equation (176) which was the first to replace the older Cockcroft-Gault equation.(177)

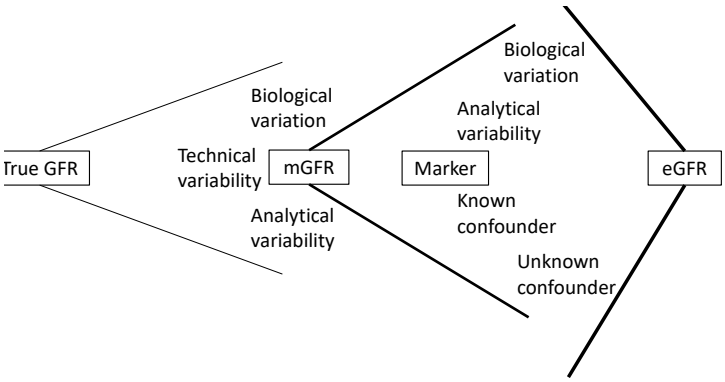

Figure 7: Relationship between true GFR, measured GFR, marker concentration and estimated GFR

Important parameters when assessing comparability and applicability of eGFR equations are therefore

- Method of gold-standard GFR measurement(13)
- Assay (incl calibration) used for marker measurement
- Mean GFR
- Age
- Race
- Gender
- Underlying disease
- Other known confounders.

Clearly, the size of the development cohort affects the robustness of coefficients found and nowadays often widely exceeds 1,000 individuals. In children, however, numbers are often smaller due to the invasiveness of the gold standard GFR measurement. Some high-risk populations, such as premature babies may not be available for such studies at all. Although there are a number of studies exploring endogenous markers in premature infants, (178) only very few used a gold standard clearance, all of which with exceedingly low participant numbers.(179)

Performance of individual eGFR equations is judged by several parameters: (i) Bias (i.e. mean or median of the difference between eGFR and mGFR) or %prediction error (i.e. mean or median (eGFR – mGFR)/ mGFR x 100%), (ii) precision measured as the scatter of the bias or as absolute %prediction error (i.e. mean or median |(eGFR – mGFR)|/ mGFR x 100%) and (iii) accuracy P_x describing the proportion of eGFR results within ± X % of mGFR. Bias and accuracy of the eGFR compared to mGFR are visualized using Bland-Altman

graphs plotting the index mGFR vs the difference of mGFR and eGFR.(180) Performance in the classification according to CKD stages can be assessed using receiver-operating characteristics (ROC) plots. (181)

To be acceptable for clinical use, P_{30} accuracy should be at least 80%. (13) This means that at an eGFR of 100 ml/min/1.73m^2, there is a 20% probability of mGFR being below 70 or above 130 ml/min/1.73m^2). Modern pediatric eGFR equations (112) have P_{30} around 90% and P_{10} up to 45%. This illustrates the shortcomings of eGFR and the need to perform mGFR measurements when exact knowledge of GFR is required. It should be borne in mind, however, that part of this imprecision reflects variability in mGFR measurement rather than the eGFR. As shown by Soveri et al, (13) P_{30} of different mGFR methods compared to renal inulin clearance – the "true" gold standard - ranged from 86 to 97% with P_{10} of 50 to 72%. Also, in 8% of repeated mGFR measurements in an adult population, the difference between both tests exceeded 30%.(182) Therefore, agreement between mGFR and the optimal eGFR equation cannot exceed the rates reported in Soveri's meta-analysis,(13) i.e. P_{30} above 90% and P_{10} above 50 to 60%. While the former goal has been achieved by the most sophisticated equations, the latter has not been reached yet. Andersen et al, relying on a smaller reported difference between mGFR measurements in a pediatric population, (183) estimate the maximum P_{10} accuracy rate that can be achieved to be 86%. (74) However this does not account for the added imprecision for each additional variable. The realistic maximum accuracy is therefore likely lower.

Another aspect to be considered when looking at eGFR is the reciprocal relationship between the concentration of the GFR marker and mGFR (Figure 3). Most eGFR equations were established and perform best in patients with a moderate degree of renal dysfunction, while these equations fail in patients with normal GFR. (174) Laboratory variability at marker concentrations in the low normal range (a typical finding with serum creatinine in children) results in exaggerated variability of eGFR. This also precludes obtaining the diagnosis of increased GFR ("hyperfiltration") using eGFR equations,(184) particularly in children with low muscle mass.(185)

3.2 Statistical approach to the development of eGFR equations

The first eGFR equations in children were established using univariate linear regression relating the reciprocal of the marker concentration to mGFR.

(i) $eGFR = a + b \bullet (y/x)$
where y is either 1 or height, x is the marker concentration and a and b are the respective coefficients calculated by linear regression analysis.

As height is most closely related to muscle mass in children and adolescents, height was introduced to correct for changes in creatinine production with growth (45, 186-188) while for the low molecular weight proteins 1 is used.(145, 189-191)
This was extended to multiple linear regression allowing additional covariates to be included in the equation.(187, 192, 193)

(ii) $eGFR = a + b \bullet (y/x) + c \bullet z$
where y is either 1 or height, x is the marker concentration, z is another covariate such as age or weight and a, b and c are the respective coefficients calculated by linear regression analysis.

As the relationship between mGFR and the reciprocal of the marker concentration is not perfectly linear, more recent approaches performed multiple linear regression analysis on logarithmic data.(112, 115, 194-197)

(iii) $\ln(mGFR+E) = \ln(a) + b \bullet \ln(y/x) + c \bullet \ln Age$
which can be transformed to

$eGFR = a \bullet (x/y)^{b} \bullet Age^{c} + E$
where y is either 1 or height, x is the serum concentration of the marker, a, b and c are the products of linear regression and E is a fixed term for extra-renal elimination of the marker.(115)
Recent adult eGFR equations also include knots, i.e. different exponents at different marker concentrations, (198, 199), while other equations use different exponents in specific diagnosis groups.(200)

Pottel uses an entirely different approach for the development of eGFR equations. His concept is based on the idea that median GFR in a healthy population corresponds to the median marker concentration in the same population, which in case of creatinine may change considerably with growth reflecting changes in body composition.(44) Using age-specific reference values for IDMS-calibrated creatinine at one-year intervals, he estimates GFR by multiplying median normal GFR (i.e. 107.3 ml/min/1.73m^2 for children older than two years) with the ratio of median normal creatinine for age and sex (Q) and the observed creatinine.(201, 202)

(iv) $eGFR = a_i / (x_i/Q_i)$

Where a_i is the median GFR found in the healthy peer population of individual, x the individual's marker concentration and Q_i the median reference value in the healthy peer population

This approach has been externally validated for children and performed comparably to the height-dependent Schwartz equation. (190, 203) By using narrow age-related reference values in other age groups, this concept has been extended to the full age-spectrum.(204) This also solves the problem of discrepant results yielded by different eGFR equations (i.e. the pediatric Schwartz compared to the adult CKD-Epi or MDRD equations) at transition from adolescence to young adulthood.(205)

3.3 Standardization of measurements

One of the reasons for the wide array of eGFR equations using the same parameters lies in a lack of standardization both of the mGFR measurement (13, 14, 206) and the measurement of the endogenous markers. Considerable efforts have been made to standardize creatinine (35, 207) and cystatin C measurement.(114, 115) Since standardization reproducibility of equations has improved.(208-210)

3.4 eGFR equations in children

During the last 30 years, a large number of different eGFR equations have been developed for children using one or several of the endogenous markers of GFR discussed in this review. A selection of equations, which were developed at least in part in pediatric populations is presented in Table 2.(112, 115, 141, 145, 191, 196, 197, 201, 202, 204, 211-218) With respect to creatinine and cystatin C, we have restricted our selection to equations using current IFCC calibrated assays in order to provide clinically useful rather than historical information. More equations have been developed in adult populations, some of which have been shown to perform well in pediatric populations as well.(199)
One consideration regarding the use of eGFR equations in children is the need of anthropometic data, i.e. height, for most creatinine-based equations. These data are not readily available to the clinical laboratory, which forms an obstacle to direct reporting of eGFR by the laboratory. Here, the height-independent Pottel approach using the FAS-age equation and cystatin C based equations is clearly advantageous.(49)

Table 2: eGFR equations based on different endogenous markers
eGFR; estimated glomerular filtration rate, sCr; serum creatinine concentration, sCys; serum cystatin C concentration, B2M; serum beta-2 microglobulin concentration, BTP; serum beta-trace protein concentration, PENIA; particle enhanced nephelometric immune assay, PETIA; particle enhanced turbidimetric immune assay, CKD; chronic kidney disease, GN; glomerulonephritis, Tx; transplanted, NS; nephrotic syndrome, DM; diabetes mellitus.

Study	Equation	Assay	Population	Gold standard used	GFR (ml/min/1.73m^2)	P_{30} accuracy
Creatinine based						
Schwartz 2009 (211)	eGFR= 0.413 x height (cm)/sCr (mg/dL)	Enzymatic	349 children; 1-16 years; mild to moderate CKD; 20% GN, 73% non-GN, 7% other	Iohexol plasma clearance	41.3	79.4%
Hari 2012 (212)	eGFR=0.42 x height (cm)/sCr (mg/dL)	Jaffe	197 children; 2-18 years; CKD stages I-IV; 83 % urological disease, 10 % GN	^{99m}Tc-DTPA plasma clearance	80.5	71.4%
Pottel 2012 (201)	eGFR=107.3/(sCr/Q) Q= age- or height-based normal value	Enzymatic below age 5 years, Jaffe above	353 children; 1.6-14 years; healthy	^{51}Cr-EDTA plasma clearance	104	72.8%
Schwartz 2012 (112)	eGFR = 0.423 x [height (cm)/sCr (mg/dL)]$^{0.79}$	Enzymatic	643 children; 1-16 years	Iohexol plasma clearance	43.3	80.4%
De Souza 2012 (216)	eGFR = k x height (cm) /sCr (mg/dL), where k = 0.325 for < 13 years, 0.365 for boys > 13 years and 0.325 for girls > 13 years	Jaffe	360 children; renal transplant, other transplants, glomerular disease, CKD	Inulin urinary clearance	86.0	91%
Nagai 2013 (213)	eGFR = 0.35 x height (cm)/sCr (mg/dL)	Enzymatic	174 children; 2-11 years; mostly urological disease	Inulin urinary clearance	65.4	n.a.
Hoste 2014 (202)	eGFR = 107.3/(sCr/Q) Q = age- or height-based normal value	Enzymatic	15 978 healthy children from 0.1-20 years. Validation in 418 children	Inulin urinary clearance (Validation)	n.a.	91.2%
Uemura 2014 (190)	eGFR = 110.2 x sCr_{ref}/sCr (mg/dL) + 2.93 sCr_{ref} based on height	Enzymatic	131 children; 1 month-18 years mostly urological disease	Inulin urinary clearance	58.8-74.6	82%
Pottel 2016 (204)	eGFR = 107.3/(sCr/Q) Q = age- or height-based normal value	Enzymatic	Validation in 735 children and 1764 adults, healthy individuals	Iohexol plasma clearance, inulin urinary clearance, iothalamate plasma clearance	94.5	87.5%
Millisor 2017 (197)	eGFR = 0.33 x (height (cm) /sCr (mg/dL))	Enzymatic	299 children with malignancy	^{99m}Tc-DTPA plasma clearance	105 pat < 60 194 pat > 60	76.33%

Study	Equation	Assay	Population	Gold standard used	GFR (ml/min/1.73m^2)	P_{30} accuracy
Cystatin C based						
Schwartz 2012 (112)	eGFR= 40.6 x (1.8/sCys (mg/L/1.17)$^{0.93}$	PENIA	643 children; 1-16 years	Iohexol plasma clearance	43.3	82.6%
Uemura 2014 (214)	eGFR = 104.1 x 1/ sCys (mg/L) - 7.80	PETIA	135 children; 1 month-18 years, mostly urological disease	Inulin urinary clearance	66.3	84%
Grubb 2014 (115) (CAPA)	eGFR= 130x sCys (mg/L)$^{-1.069}$ x age (years)$^{-0.117}$-7	6 different PENIA/PETIA assays	2708 adults and 456 children	Iohexol plasma clearance, inulin urinary clearance, inulin plasma clearance	Adults 53-54 children: 103	80.1%
Berg 2015 (217)	eGFR = 91 x sCys (mg/L)$^{-1.213}$	Mostly PENIA, some PETIA	220 children; 4-18 years; various renal diseases (Tx, NS, GN, DM)	Inulin urinary clearance	84	86%
Pottel 2017 (215)	eGFR = 107.3 x sCys (mg/L) / 0.82	Different PENIA/ PETIA	5764 adults and 368 children	Various	n.a.	86.1%
B2M based						
Ikezumi 2015 (191) (183)	eGFR = 149 x (1/B2M (mg/L)) +9.15	PETIA	174 children; 1 month-18 years; mostly urological disease	Inulin urinary clearance	71.8	n.a.
BTP based						
Abbink 2008 (145)	eGFR = -35.20 + 122.74 x BTP (mg/L)$^{-0.5}$	PENIA	85 children, mean age 9.7 years, 35 oncology, 50 nephrology patients	Inulin plasma clearance	90.2	65.6-81.6%
Benlamri 2010 (196)	eGFR = 79.8 x [1/BTP (mg/L)]$^{0.9515}$	PENIA	474 children; 0.21-18.9 years Various renal pathology	^{99m}Tc-DTPA plasma clearance	105.5	76.8%
Combined						
Schwartz 2012 (117) (CKiD3)	eGFR = 39.1 x [height (cm)/sCr (mg/dL)]$^{0.516}$ x [1.8/sCys (mg/L)]$^{0.94}$ x [30/BUN (mg/dL)]$^{0.169}$ x 1.099$^{1 \text{ if male}}$ x [height (cm)/1.4]$^{0.188}$	Enzymatic& PETIA	643 children; 1-16 years	Iohexol plasma clearance	41.3	91.0%

Chehade 2014 (210)	For boys: eGFR = 0.42 x (height/sCr) – 0.04 x (height/sCr)^2 – 14.5 x sCys + 0.69 x age + 21.88 For girls: eGFR= 0.42 x (height/sCr) – 0.04 x (height/sCr)^2 – 14.5 x sCys + 0.69 x age +18.25	Jaffe &PENIA	243 children; 2-18,5 years. Mostly obstructive nephropathy or single kidney	Inulin urinary clearance	85	97%
Witzel 2015 (135)	For boys: eGFR= 666.8/BTP (mg/L)$^{0.461}$/ sCr (mmol/L)$^{0.679}$) x $10^{0.00259 \times \text{height (cm)}}$ For girls: eGFR = 591.6/ BTP(mg/L)$^{0.433}$/ sCr (mmol/L)$^{0.661}$ x $10^{0.00256 \times \text{height (cm)}}$	Enzymatic &PENIA	504 children; 2-18 years, various renal pathology	^{99m}Tc-DTPA plasma clearance	110	80.2-88.6%

3.5 Combination of markers

As the extra-renal metabolism of the different endogenous markers involves different tissues and pathways, it is not surprising that a combination of markers into one single eGFR equation (112, 198, 219) or the average of the results of separate one-parameter eGFR equations significantly improves predictive performance. (49, 220, 221) Both approaches yield comparable results with regard to bias and accuracy. Still, the "Lund approach" proposed by Anders Grubb comparing a creatinine- and a cystatin C- based eGFR and using the average of both estimates (222) offers several advantages over the use of more complex equations. (i) It draws the clinician's attention to discrepant results of the two estimates, which can be a clue for yet unrecognized pathology.(27, 223) (ii) In this situation a motivated choice for one of the two equations may be more accurate than using the average. (iii) Analyzing concordance between both eGFR estimates adds confidence to the accuracy of the estimates: If the difference between the estimates is less than 30% this indicates a P_{30} accuracy of the average exceeding 92% and a P_{10} accuracy exceeding 45%. (49) (iv) In case the cystatine C-based eGFR is more than 40% lower than the creatinine-based eGFR (in the absence of neuromuscular disease, high-dose glucocorticoids or untreated hypothyroidism) this may suggest the presence of the recently postulated "shrunken pore syndrome" in which changes in the size selectivity of the glomerular filtration barrier lead to retention of the larger LMW protein markers in excess of creatinine.(224) This condition has been associated with higher mortality in adults.(103)

4. Summary and conclusion

As in the adult population, estimation of GFR from endogenous markers in children is an important method to aid recognition and follow-up of renal dysfunction. Because of physiologically low creatinine levels, enzymatic assays are mandatory at younger ages, and diagnosis of early stages of renal injury may be missed. Age-related changes in creatinine production have to be considered when using this marker. Therefore, correction for age or height is necessary and has been incorporated in pediatric creatinine-based eGFR equations. Being independent of muscle mass, cystatin C can be used without anthropometric data. The combination of both markers, either in one single equation or by calculating the average of a creatinine- and a cystatin C-based eGFR improves accuracy of eGFR prediction. The role of B2M and BTP in pediatrics still needs to be defined. Their renal characteristics show considerable overlap with cystatin C, while extra-renal influences differ so that a combination with the other markers of kidney function may be

useful. When an exact measurement of GFR is required, eGFR lacks accuracy and a gold standard measurement should be performed. Here, iohexol plasma clearance is a well-characterized technique without radiation exposure.

Conflict of interest:
On behalf of all authors, the corresponding author declares that there are no conflicts of interest.

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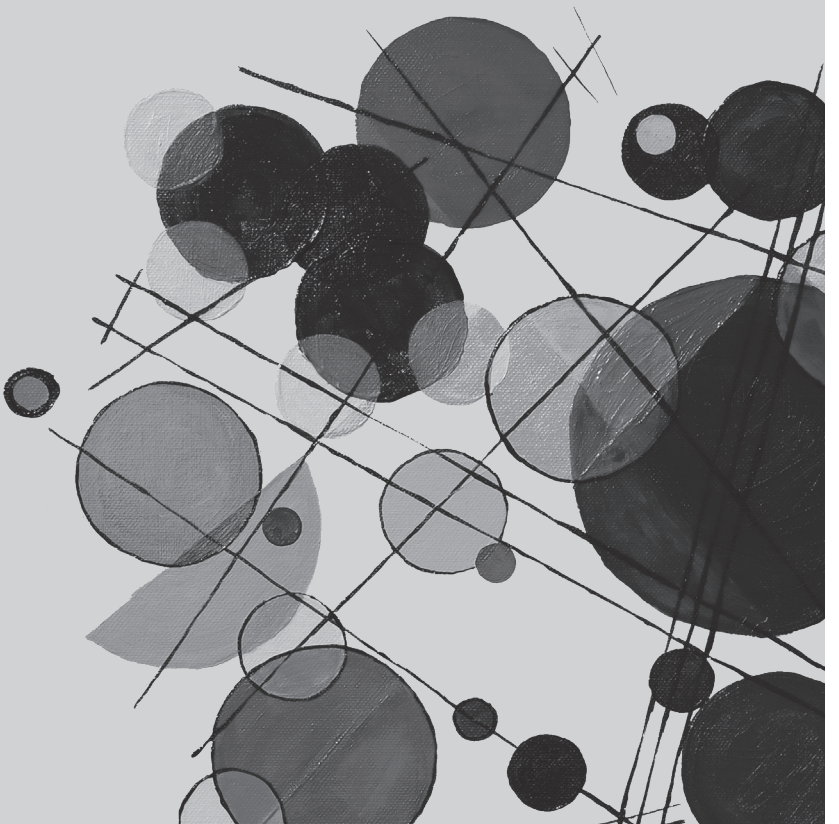

GFR-estimation by serum creatinine during glucocorticosteroid therapy

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Abstract

Background

While glucocorticosteroids (GCS) are widely used in patients with kidney disease, little is known about their effect on serum creatinine, the most commonly used endogenous marker of kidney function.

Methods

We assessed the effect of GCS on the relationship between estimated GFR using the Schwartz equation (eGFR) and measured GFR using a single injection inulin clearance (Cin) in children both in a paired analysis and a cross-sectional study. Primary outcome variable was the difference between eGFR and Cin (ΔGFR) in a paired analysis involving 22 patients during and off GCS treatment (mean GFR 103.8 ml/min/1.73m^2, mean prednisone dose 34.8 mg/m^2/d). In a cross sectional analysis in 42 patients receiving GCS (mean dose of 25.7 mg/m^2/d), a dose-dependent effect was explored using univariate and multivariate linear regression of various variables including GCS dosage with serum creatinine as dependent variable.

Results

The paired analysis showed no significant difference in ΔGFR with or without GCS (-23 [SD 53] vs. -9 [SD 41] ml/min/1.73m^2, p = 0.203). Stepwise multivariate linear regression analysis showed a significant correlation between age and Cin, while GCS dose was not related to serum creatinine.

Conclusion

GCS use had no significant effect on serum creatinine as a marker for kidney function in a mixed population of renal outpatient clinic children.

Introduction

Glucocorticosteroids (GCS) are widely used in patients with kidney disease. Serum creatinine is the most commonly used endogenous marker of kidney function. While the effects of GCS treatment on other GFR markers such as cystatin C, β-2-microglobuline and β-trace protein have been well characterized, (1-3) little is known about GCS treatment and creatinine metabolism. GCS have been shown to increase inulin clearance (Cin) in healthy persons, (1, 4) while there have been conflicting results as to GCS effects on serum creatinine and creatinine clearance. Connell et al (5) observed no change in creatinine clearance despite a significant increase in Cin following 5 days of ACTH treatment, while van Acker et al (4) found a rise in both serum creatinine concentration and creatinine excretion in patients with Graves' ophthalmopathy receiving high-dose prednisone for 2 weeks. Studies in dogs confirm a steroid-induced increase in GFR, while the effect on changes in serum creatinine was variable. (6, 7)

The aim of our study was to analyze the effect of GCS therapy on the relationship between serum creatinine and GFR measured by single-injection inulin clearance.

Materials and methods

Study subjects

Between October 2004 and March 2015, a total of 514 inulin clearance tests were performed at the department of pediatric nephrology at VU university medical centre on clinical grounds or as part of institutional review board-approved research projects. (1, 2, 8, 9) Here, we performed a retrospective analysis of the effect of GCS treatment on serum creatinine in a subgroup of this population with nephritis and malignancy. The study was conducted in accordance with the declaration of Helsinki and the waiver of informed consent was approved by the institutional review board of VU university medical centre; medisch ethische toetsingscommissie (METC).

Patients were classified as GCS-positive if they had received pharmacological doses of GCS for a minimum of 5 days prior to performance of the clearance study and as GCS-negative if they had been off GCS for at least 10 days.

The study comprised a paired analysis and a cross-sectional analysis. The paired analysis involved 22 patients who underwent two clearance studies, one with and one without GCS treatment, serving as their own controls. The cross-sectional analysis involved all patients

receiving GCS from our database including GFR measurements of the patients from the paired analysis while receiving GCS. All included patients were checked for medications known to interfere with creatinine excretion such as cimetidine, (10) trimethoprim, (11) dronedarone (12) or cobicistat,(13). With the exception of trimethoprim-sulfamethoxazole, which was prescribed to all patients with active malignancy as prophylaxis against pneumocystis pneumonia, no drugs interfering with creatinine metabolism were identified.

Measurements

GFR was measured using the single-injection inulin plasma-disappearance method, which has been proved to be an accurate method to determine GFR in children. (14) All patients received a single intravenous dose (5000 mg /1.73m² of body surface area with a maximum dose of 5000 mg) of inulin (Inutest®, Fresenius, Bad Homburg, Germany) within 1 minute. Serial blood samples were obtained at 10, 30, 90 and 240 minutes after injection. Inulin concentrations were measured in serum by an enzymatic method (15) and inulin clearance in ml/min/1.73m² was calculated with MW/Pharm 3.5 software (Mediware, Groningen, The Netherlands), a pharmacokinetic computer program using a Bayesian estimate from patient and population data. (14)

On the same day, serum creatinine was determined enzymatically with an IFCC-traceable assay (Modular analytics <P>, Roche diagnostics, Mannheim, Germany). Creatinine and height were combined to calculate estimated GFR (eGFR) using the revised Schwartz equation: (16)

(1) eGFR (ml/min/1.73m^2 ≈ height (cm) x 36.4 / serum creatinine (µmol/l)

Statistical analysis

In order to study the interaction between GCS treatment and creatinine as a marker for kidney function independently of the effect of GCS on kidney function itself, the difference between measured GFR (Cin) and creatinine-based eGFR was calculated as

(2) ΔGFR = Cin - eGFR

Changes in ΔGFR were used to analyse a potential effect of GCS in the paired analysis. Outcome data are presented as mean [standard deviation]. Data from the paired analysis were analysed using a paired samples t-test.

In the cross-sectional study the effect of age, gender, height, 1/Cin, diagnosis group (malignancy vs. nephritis) and GCS-dose and duration of therapy (short; <2 weeks, middle; 2-4 weeks or long; >4weeks) on serum creatinine as dependent variable was studied by

both univariate and stepwise multivariate linear regression analysis (forward model, F-to-enter of 4.0) to search for a dose dependant effect.
In all analyses, GCS dose was expressed as prednisone equivalent in mg/m^2/d. In case of dexamethasone treatment, the dexamethasone dose was converted into prednisone equivalents by multiplying by a factor of 6. (2)

Results

Paired analysis

The paired analysis was performed on two separate occasions, one with GCS and one off GCS in two subgroups: (i) Eleven children with nephritis, nine of whom with IgA nephropathy, one with focal segmental glomerulosclerosis (FSGS) and one with systemic lupus erythematosus (SLE), all male, mean age 14.9 years [SD 2.3], mean prednisone dose 25.0 mg/m^2/d [SD 13.6], mean time lag between both tests 237 days [SD 180] and (ii) Eleven patients with malignancy, nine of whom with lymphatic leukaemia (ALL), one with osteosarcoma and one with a primitive neuro ectodermal tumor (PNET), six males and five females, mean age 8.0 years [SD 3.6], mean prednisone dose 44.6 mg/m^2/d [SD 12.7], mean time-lag between both tests 72 days [SD 86]. All the patients with ALL were enrolled in the same treatment protocol (ALL-10 of the Dutch Childhood Oncology Group (SKION)). The results of the analysis by paired samples t-test are summarized in table 1. Neither in the total group of 22 patients, nor in the two subgroups a significant difference in ΔGFR on or off GCS was observed. These findings were confirmed if tested non-parametrically (data not presented).

Table 1: Paired analysis on and off glucocorticoid treatment
GCS-; Clearance study off glucocorticoid treatment, GCS+; Clearance study with glucocorticoid treatment. Cin; inulin clearance expressed in ml/min/1.73m^2, eGFR; estimated kidney function using Schwartz equation expressed in ml/min/1.73m^2, ΔGFR; difference between Cin and eGFR expressed in ml/min/1.73m^2. Data presented as mean [standard deviation]

		GCS -	GCS +	P
		Mean [SD]	*Mean [SD]*	
Malignancy (n=11)	Cin	117 [27]	124 [32]	0.33
	eGFR	161 [54]	149 [44]	0.48
	ΔGFR	-44 [65]	-25 [42]	0.33
Nephritis (n=11)	Cin	91 [30]	105 [27]	0.13
	eGFR	93 [31]	98 [26]	0.54
	ΔGFR	-2 [26]	7 [35]	0.44
Total (n=22)	Cin	104 [31]	114 [30]	0.07
	eGFR	127 [56]	124 [44]	0.71
	ΔGFR	-23 [53]	-9 [41]	0.20

Cross-sectional analysis using serum creatinine as outcome parameter

We combined all the patients receiving GCS from the paired analyses and 20 additional nephritis patients, eight of whom with FSGS, three with SLE, three with IgA nephropathy, three with membranoproliferative glomerulonephritis (MPGN), one with minimal change nephrotic syndrome, one with microscopic polyangiitis and one with IgM nephropathy who were only tested during GCS treatment yielding a group of 42 patients. Mean age was 12.5 years [SD 4.1], 59.5% were male, mean GFR was 95.5 ml/min/1.73m^2 [SD 33.2] and mean steroid dose was 25.7 mg/m^2/d [SD 20.4]. The results of univariate regression analysis are presented in table 2. Here, all tested parameters but gender were significantly related to serum creatinine. After correction for confounding factors using stepwise multiple variate regression analysis of all the parameters, which were statistically significant by univariate analysis, only 1/Cin and age were retained in the final model. The correlation coefficient for GCS-dose in mg/m^2/d was -0.131, which was not statistically significant (p-value 0.104). Table 2

Table 2: Cross-sectional analysis. Univariate linear regression analysis with serum creatinine in µmol/l as dependant variable. Independent variables listed, for gender 0 is male, 1 is female, for diagnosis 0 is nephritis, 1 is malignancy, duration of steroid treatment is divided into short, middle and long. Data presented as B [95% confidence interval] along with p-value.

	B [95% CI]		P
Age (yrs)	4.030	[2.221 to 5.840]	<0.001
Gender (0;M, 1;F)	7.089	[-10.984 to 25.161]	0.433
1/Cin (1/(ml/min/1.73m^2))	3471	[2527 to 4414]	<0.001
GCS dose (mg/m^2/d)	-0.685	[-1.071 to -0.299]	0.001
Height (m)	0.699	[0.345 to 1.053]	<0.001
Weight (kg)	0.648	[0.216 to 1.079]	0.004
Diagnosis (0; N 1; M)	-39.861	[-55.710 to -24.012]	<0.001
Duration steroid treatment	19.298	[10.266 to 28.329]	<0.001

Discussion

This is the largest study of the effect of GCS on serum creatinine as a marker of GFR in humans and the first in children. In contrast to all previous studies we used ΔGFR as the difference between creatinine-based eGFR and Cin, which allowed for a quantitative analysis of the interaction between GCS and serum creatinine *independent* of GCS-induced changes in inulin clearance in a paired study. This is important, as high-dose GCS have been shown to increase GFR intrinsically (1, 4) or as part of their therapeutic effect in patients with nephritis.

Our data show no significant effect of GCS treatment on ΔGFR in the paired analysis nor on serum creatinine in the multivariate cross-sectional analysis. This indicates that GCS have no strong effect on serum creatinine levels after correction for changes in GFR.
Looking at the paired analysis in detail, ΔGFR in the malignancy group was negative both on and off GCS treatment indicating overestimation of GFR by serum creatinine, which has been shown previously. (8) Cin tended to be higher in both the nephritis and the malignancy group during glucocorticoid treatment, most likely reflecting a GCS-induced increase in GFR as observed in several studies both in man (1, 4) and in dogs. (6, 7) In the nephritis group, a positive therapeutic effect on glomerular function may also have played a role.

The cross-sectional analysis revealed only an association of serum creatinine with age and 1/Cin, both of which are to be expected from the physiology of serum creatinine in children. (17) The absence of a gender effect on serum creatinine in this analysis is remarkable and cannot be explained.

Our study has several limitations. (i) Although the largest study in humans to date, due to the retrospective nature and the invasiveness of the inulin clearance test, numbers are low and power is limited. Therefore, a moderate effect of GCS on serum creatinine may have been missed. (ii) Although eGFR is the clinical standard for reporting kidney function in daily practice, its accuracy is limited. Only around 75% of estimates are within ±30% of a gold standard GFR measurement in children. (16) As the inaccuracy of eGFR introduces extra variability when using ΔGFR as outcome parameter, we chose to analyse the effect of GCS on crude serum creatinine in the cross-sectional analysis. Neither the paired analysis using ΔGFR nor the cross-sectional analysis using serum creatinine revealed a potential interaction. Of note, ΔGFR tended to be higher in the paired analysis during GCS treatment indicating an *under*estimation by creatinine-based eGFR. In the cross-sectional analysis, however, the opposite is observed: a trend towards a negative B-value with increasing steroid dose suggesting progressive *over*estimation of GFR. (iii) In the paired analysis both duration of GCS treatment and GCS dose were variable reflecting differences in patient population and treatment protocols between nephritis and oncological patients. There was a significant time lag between measurements with and without steroids in the nephritis group in the paired analysis, so that changes in body composition between both tests unrelated to GCS treatment cannot be excluded. In the cross-sectional analysis, both GCS dose and duration of GCS treatment were not significantly related to ΔGFR, however. (iv) Apart from GCS the patients with malignancy also received trimethoprim-sulfamethoxazole, a drug which is known to interfere with tubular creatinine secretion. As trimethoprim-sulfamethoxazole was administered during

both tests in the paired analysis this should not have affected our results. (v) 24-hour creatinine excretion was not measured in this population due to the retrospective design and the well-documented unreliability of this measurement, in particular in children. It might have provided information about the production and the renal clearance of creatinine, explaining a potential effect of GCS on serum creatinine.

In conclusion GCS use had no significant effect on serum creatinine as a marker for kidney function in a mixed population of renal outpatient clinic children.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study formal consent is not required.

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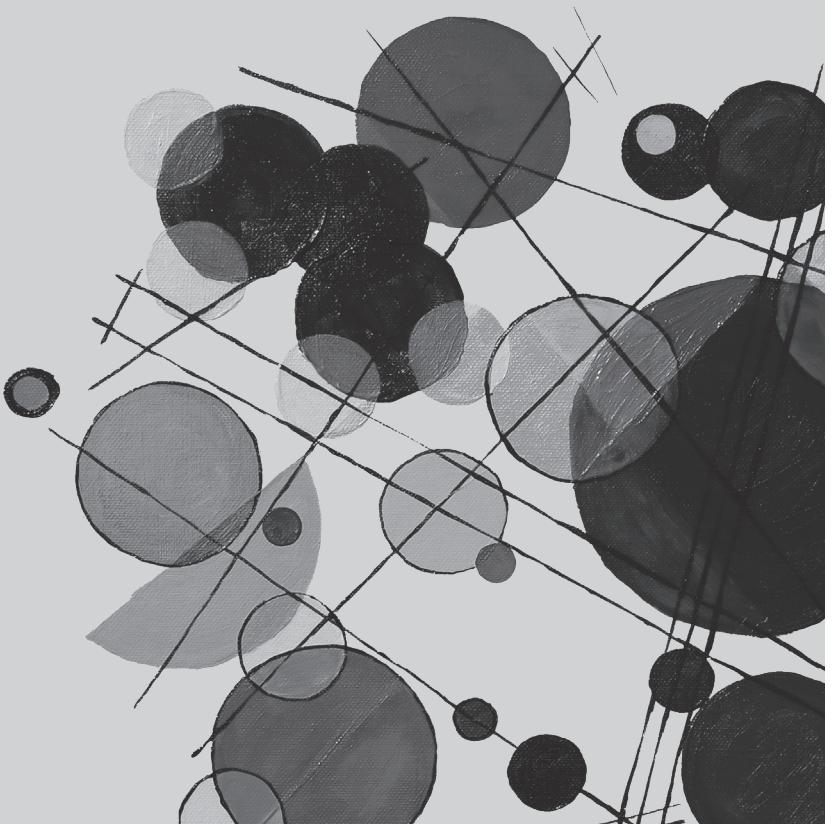

Estimation of GFR in children using rescaled beta-trace protein

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Abstract

Introduction

Beta-trace protein (BTP) is a low molecular weight protein, produced mainly in the cerebrospinal fluid. It has been proposed as a marker for kidney function. Recently, a new method for GFR estimation using mean normal values to rescale GFR marker concentrations has been described for creatinine and cystatin C, two commonly used endogenous markers for kidney function. The aim of this study is to apply this approach to BTP in children.

Method

We retrospectively analyzed serum concentrations of creatinine, cystatin C and BTP measured during inulin clearance tests in children. BTP was measured using a particle-enhanced immunonephelometric assay (Siemens Healthcare). A novel BTP-based eGFR equation was developed using published normal values for children: $eGFR_{BTP}$[ml/min/1.73m^2]=107.3/BTP/Q_{BTP} with Q_{BTP} = 0.69. Performance of this equation was compared to the established creatinine-based full age spectrum equation FASage and the cystatin C-based FAScys equations as well as the BTP-based Benlamri equation in terms of bias, % prediction error and P_{30} and P_{10} accuracy rates.

Results

322 inulin clearance tests were studied. Overall, our novel equation performed comparably to the creatinine-based FASage and the BTP-based Benlamri equations but was less accurate than FAScys (P_{30}: 79.2 vs 86.3%, p=0.008). Combining markers significantly enhanced performance compared to the single marker equations, with the exception of FAScys.

Conclusion

Rescaled BTP concentrations are a simple method for estimating GFR in children. However, the additional value of BTP for the estimation of GFR compared to rescaled creatinine and cystatin C still remains to be demonstrated.

Introduction

Beta-trace protein (BTP) is a low molecular weight protein (molecular weight ranging from 20 to 31 kDa) with a carbohydrate residue on the N-terminal end accounting for the large variance in molecular weight (1). It is produced primarily in the central nervous system by glial cells, leptomeningeal cells and in the choroid plexus (2, 3). Therefore, highest concentrations are found in cerebrospinal fluid (4, 5). BTP leaks from the cerebrospinal fluid into the serum, where significantly lower concentrations are observed (6). The liver eliminates BTP with shorter residues, resulting in distinct cerebrospinal fluid and serum glycosylation patterns. Therefore, serum BTP has a higher molecular weight and the weight distribution is narrower (26-29 kDa) (6-8). Like other low-molecular weight proteins, serum BTP is almost exclusively eliminated through glomerular filtration and degraded in the proximal tubules (9). Therefore serum BTP has been proposed as a marker of GFR in both children and adults (10-12).

In order to convert serum levels of an endogenous GFR marker to an estimate of GFR, marker-specific eGFR equations are required (13). Usually, these equations are calculated using some form of linear regression analysis (11, 14-16). A major disadvantage of this method is that unknown confounding factors in the calibrating population will alter the slope of the regression (13). Moreover, in order to create an equation that is applicable over the full spectrum of GFR, subjects comprising the whole range of GFR must be present in the population, including patients with very low GFR. Many of such patients have severe co-morbidity, adding to the impact of unknown confounding factors. The distribution of GFR in a nephrology unit usually peaks at CKD stages 3 to 4 and fewer patients with mildly impaired or more advanced renal failure are included in the large cohorts used to create the most widespread current eGFR equations (CKD-EPI, mean GFR 70 ml/min/1.73m^2 (17); MDRD, 40 ml/min/1.73m^2 (18); CKiD, 43 ml/min/1.73m^2 (15).

Recently, Pottel et al. (19) developed an alternative strategy to create eGFR equations by using appropriate normal values to rescale the marker concentration in an individual patient. These normal values are derived from healthy populations where GFR is normal. Due to changes in muscle mass during growth and development, creatinine reference values change rapidly and separate gender-specific reference values (Q_{crea}) are needed (19, 20). This is not the case for cystatin C, where age, height or sex have no (13) or only mild (21) effects in the pediatric age range, resulting in a constant reference range and thus a single Q value (22).

Rescaling serum levels to individual reference values has several advantages (23). (i) It makes the different markers easier to compare. While serum creatinine levels differ from serum cystatin C levels, rescaled creatinine levels and rescaled cystatin C levels should be very similar. (ii) Rescaled serum markers follow a normal distribution with a mean of 1. Therefore, the further the rescaled marker deviates from 1, the further the eGFR will be from normal. (iii) Since differences in age, height or sex are incorporated in the Q values, the rescaled serum levels are independent of these factors, and the resulting equations can be used across the full age spectrum.
The eGFR equations following this approach have been shown to perform well in diverse pediatric populations (19, 22, 24-26). For BTP, a similar approach yielded results in elderly patients suggesting that a FASBTP equation would perform similarly (27).
The aim of this study is to apply this method in children and develop an eGFR equation using rescaled serum concentrations of BTP, which can be incorporated in an equation for the full age spectrum (FAS) and compare this equation to existing eGFR equations.

Methods

Data collection
Retrospective analysis was carried out in 322 inulin plasma clearance tests (Cin) performed in 322 children on clinical grounds over a period of 11 years. During the clearance study, blood had been taken for the measurement of serum creatinine, cystatin C and BTP. Height, weight and primary diagnosis were extracted from the patient charts, as was use of glucocorticosteroids.

Inulin clearance was measured by intravenous administration of 5000 mg/1.73m^2 body surface of inulin (Inutest®, Fresenius, Bad Homburg, Germany) with a maximum of 5000 mg. Subsequently, serum samples were taken 10, 30, 90 and 240 minutes after injection and inulin concentrations measured using an enzymatic method (28). Clearance was calculated from the decline in serum concentration using MW/Pharm 3.5 software (Mediware, Groningen, The Netherlands), a pharmacokinetic computer program using a Bayesian estimate from patient and population data (29). This method has been described previously in more detail (24).

Between March 2008 and September 2014 creatinine was measured using an IDMS traceable creatinase/sarcosine oxidase enzymatic method on the Modular P800 chemistry analyzer (Roche Diagnostics, Mannheim, Germany). The intraassay coefficient of variation for creatinine was 0.7% (mean = 1.21 umol/L, n = 10), whereas the interassay coefficient

of variation (CV) was 1.8% (mean = 368 umol/l; n = 10). Creatinine results from September 2014 onwards were measured using the same IDMS traceable assay on the Cobas8000 chemistry analyzer (Roche Diagnostics, Mannheim, Germany; interassay CV= 1.5% , mean 80 umol/l and 1.4%, mean 593 umol/l (Unity Real Time QC, Biorad). For measurements before 2008, a kinetic Jaffe reaction was used (Modular P800, Roche Diagnostics, Mannheim, Germany). These measurements were corrected to fit IDMS traceability by an equation developed locally [IFCC creatinine (µmol/l) = Jaffe creatinine x 1.1 – 26].

Both serum cystatin C and serum BTP levels were measured using particle enhanced immunonephelometric assays (N Latex CYSC™ and N Latex BTP™, Siemens Healthcare Diagnostics, Eschborn, Germany) on a Behring Nephelometer II. For cystatin C the assay was calibrated to fit IFCC reference material (ERM®-DA471/IFCC). For data collected prior to the use of this reference material from 2013 onwards, a conversion factor of 1.17 was used as recommended by the manufacturer. The intraassay coefficient of variation for BTP was 2.8% (mean = 1.6 mg/l; n = 10), whereas the interassay coefficient of variation was 3.7% (mean = 1.7mg/l; n = 10). The intraassay coefficient of variation for cystatin C was 2.9% (mean = 1.1 mg/l; n = 10), whereas the interassay coefficient of variation was 3.4% (mean = 1.3mg/l; n = 10).

Characteristics of BTP in a subset with normal renal function

From our database, a subset of unique patients was selected to estimate the normal BTP concentration and assess the influence of age, height, weight, BMI and sex on the serum levels of BTP. In order to qualify for inclusion in this dataset measured GFR had to be above 90 ml/min/1.73m^2. Exclusion criteria were active malignancy, nephritis, glucocorticoid use and neural tube defects.

Development and evaluation of a BTP-based eGFR equation

The BTP-based eGFR equation was constructed using the mean BTP concentration found in healthy children from a different population where BTP had been measured in the same laboratory with the same nephelometric assay (2). In analogy to the FAScys and FAScrea (23) the equation for $eGFR_{BTP}$ was:

(i) $eGFR_{BTP}$ [ml/min/1.73m^2]= 107.3 [ml/min/1.73m^2]/S_{BTP}/Q_{BTP}
with S_{BTP} in mg/l and Q_{BTP}; 0.69 mg/l

The performance of this new equation was compared to the BTP-based equation described by Benlamri et al (30).

(ii) $eGFR_{Benlamri}$ [ml/min/1.73m^2] = $10^{(1.902+(0.9515 \times \log(1/BTP)))}$
with BTP in mg/l

as well as the creatinine-based full age spectrum FAScrea equation (19).

(iii) FAScrea [ml/min/1.73m^2]= 107.3 [ml/min/1.73m^2]/Scr/Q_{crea}
with Scr in mg/dl and Q_{crea} being the age-related normal value of creatinine in mg/dl

and the cystatin C-based full age spectrum (FAScys) equation (22).

(iv) FAScys [ml/min/1.73m^2] = 107.3 [ml/min/1.73m^2]/Scys/Q_{cys}
With Scys in mg/l and Q_{cys}; 0.82 mg/l being the normal value of cystatin C.

Finally, we also compared the individual equations to the arithmetic [i.e. ($eGFR_a$ + $eGFR_b$)/2] and geometric [i.e. ($eGFR_a$ x $eGFR_b$)$^{0.5}$]means calculated from $eGFR_{BTP}$, FAScrea and/or FAScys.
The performance in terms of %prediction error and P_{30} and P_{10} accuracy were compared across diagnosis groups, levels of measured GFR, glucocorticosteroid (GCS) use,sex and age. For levels of measured GFR, we also calculated the percentage of cases in which the eGFR equation correctly identified the CKD level (% correctly identified CKD).

Statistical analysis
We used the following parameters to explore performance of the different eGFR equations. (i) Bias was defined as eGFR - Cin (ii) %prediction error as 100x(eGFR-Cin)/Cin in %, absolute %prediction error as 100x(|Cin-eGFR|)/Cin in %. P_{30} and P_{10} accuracy describe the percentage of cases where eGFR was within ± 30% or ± 10% of Cin.

Continuous variables were analyzed using Spearman's correlation. For the dichotomous variable sex, an independent samples T-test was used. Accuracy rates were compared using McNemar tests.

Results

Population characteristics

Over a period of 11 years, 322 inulin clearance tests with simultaneous BTP and creatinine serum levels were documented in 322 unique patients. Patient age ranged from 2.1 to 19.5 years with a median of 14.2 years, median GFR measured by inulin clearance was 94.3 ml/min/1.73m^2 with a range from 13.4 to 185.0 ml/min/1.73m^2. Further characteristics are summarized in Table 1.

Of the 322 measurements, 91 cases fit the inclusion criteria for the group with normal renal function. Here, median age was 15.5 years (range 2.3 to 19.5), 54.9% were male, median GFR was 100.0 ml/min/1.73m^2 and median serum BTP levels was 0.730 mg/l. Further characteristics of this group with normal renal function are summarized in Table 1.

Table 1: Patient characteristics of the healthy subgroup and the total dataset
Data are given as median [interquartile range] or as percentage.

		Healthy subgroup		Total dataset	
Number		91		322	
GFR (ml/min/1.73m^2)		100.0	[94.1 to 110.7]	94.3	[76.7 to 109.8]
%males		54.9%		59%	
Height (m)		164.0	[141.4 to 175.0]	158.0	[133.9 to 171.5]
Weight (kg)		56.5	[35.0 to 69.0]	50.5	[30.9 to 64.0]
BMI (kg/m^2)		20.2	[16.9 to 23.2]	19.2	[16.7 to 22.4]
Age (years)		15.5	[10.8 to 17.7]	14.2	[9.4 to 17.4]
Serum creatinine (mg/dl)		0.69	[0.52 to 0.86]	0.69	[0.48 to 0.91]
Serum cystatin C (mg/l)		0.96	[0.84 to 1.03]	1.01	[0.87 to 1.22]
Serum BTP (mg/l)		0.73	[0.64 to 0.82]	0.77	[0.65 to 0.99]
Diagnosis	Malignancy	0%		19.6%	
	Single kidney	52.7%		28.6%	
	Nephritis	0%		14.0%	
	Urological	15.4%		10.9%	
	Spina bifida	0%		9.0%	
	Follow-up after malignancy	6.6%		4.0%	
	Other	25.3%		14.0%	

None of the factors studied with Pearson's correlation had a significant effect on BTP concentrations (Table 2). However gender did have a significant effect on BTP concentrations (median 0.745 mg/l in males vs 0.690 mg/l in females, p=0.025).

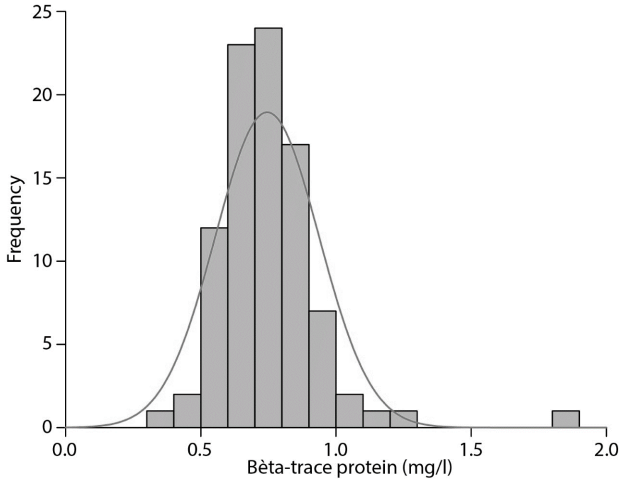

Figure 1: Distribution of serum levels of BTP in our healthy subgroup

We used median BTP concentrations in this analysis, since the data in our healthy subgroup group were not normally distributed (Figure 1; Shapiro-Wilk test for normality 0.813, p= 0.000), furthermore due to the relatively small reference group, outliers would have more impact on the mean.

Table 2: Factors potentially related to the serum concentrations of BTP in the healthy subgroup

	Spearman's correlation (p-value)
Age	-0.165 (p=0.119)
Height	-0.098 (p=0.357)
Weight	-0.122 (p=0.249)
BMI	-0.122 (p=0.249)

eGFR equation

We tested the new $eGFR_{BTP}$ equation in terms of bias, %prediction error, |%prediction error| and P_{30} and P_{10} accuracy rates (Table 3). Overall, $eGFR_{BTP}$ performed comparably to the creatinine-based FASage and the BTP-based Benlamri equations and was significantly less accurate than FAScys (P_{30}: 79.2 vs 86.3%, p=0.008). Combining equations as geometric or arithmetic mean led to improvement in all outcome parameters. The combination of all three performed best with significantly higher P_{30} and P_{10} accuracy rates compared to any of the three single marker equations, with the exception of FAScys, for which the

increased accuracy did not reach statistical significance. Combining all three markers did not improve accuracy when compared to pairs of any two of the eGFR equations.

Table 3: Performance of $eGFR_{BTP}$ versus FAScrea
FAScys and the Benlamri equation in terms of bias, % prediction error, |%prediction error|. Data presented as in median [interquartile range] ml/min/1.73m^2. Accuracy presented as P_{30} and P_{10} in %.

	Bias	% prediction error	|%prediction error|	P_{30} accuracy	P_{10} accuracy
Benlamri	5.8 [-5.5 to 22.1]	7.1 [-6.2 to 25.0]	14.4 [6.5 to 28.0]	78.0	37.6
$eGFR_{BTP}$	-0.2 [-11.2 to 15.8]	-0.2 [-12.3 to 18.1]	15.0 [6.9 to 25.5]	79.2	33.9
FAScys	-5.1 [-17.2 to 4.3]	-4.8 [-17.5 to 6.2]	13.7 [5.3 to 22.4]	86.3	40.7
FAScrea	3.8 [-6.2 to 16.9]	4.8 [-9.8 to 21.3]	13.9 [6.7 to 24.7]	80.4	35.1
Arithmetic means					
$eGFR_{BTP\text{-}crea}$	2.7 [-5.9 to 14.1]	3.1 [-7.5 to 18.0]	11.7 [5.1 to 22.9]	82.6	45.0
$eGFR_{BTP\_cys}$	-1.6 [-11.7 to 8.4]	-1.9 [-13.5 to 10.4]	12.0 [4.7 to 21.6]	87.0	41.9
$eGFR_{crea\_cys}$	-0.8 [-9.7 to 9.6]	-0.9 [-9.9 to 13.4]	11.3 [4.6 to 19.5]	87.3	47.2
$eGFR_{BTP\_cys\_crea}$	0.3 [-8.7 to 11.3]	0.3 [-9.3 to 13.5]	11.0 [5.4 to 20.7]	87.6	45.0
Geometric means					
$eGFR_{BTP\_crea}$	2.1 [-6.4 to 13.3]	2.4 [-8.3 to 16.8]	11.5 [5.2 to 22.4]	83.5	45.0
$eGFR_{BTP\_cys}$	-1.8 [-12.6 to 7.8]	-2.3 [-13.7 to 9.8]	12.0 [4.5 to 21.3]	86.7	42.9
$eGFR_{crea\_cys}$	-1.1 [-10.6 to 9.0]	-1.2 [-10.7 to 13.0]	11.6 [4.7 to 19.7]	88.8	45.3
$eGFR_{BTP\_cys\_crea}$	-0.1 [-9.8 to 9.5]	-0.2 [-10.2 to 12.1]	10.7 [5.6 to 20.0]	87.9	46.0

Analyzing different categories of measured GFR (Table 4), performance of $eGFR_{BTP}$, and the Benlamri equation declined progressively with decreasing GFR, while accuracy of FAScrea and FAScys was comparable in CKD 1 and CKD 2. Performance of FAScys at GFR<60 ml/min/1.73m^2 tended to be higher than FAScrea and Benlamri, but this did not reach statistical significance due to small numbers in this subgroup. This was also reflected in the more consistent percentages in which the correct CKD group was identified for patients using the FAScys equation.

The performance of the equations in different diagnosis groups is presented in Table 5. Here, FAScys clearly outperformed the other equations in patients with spina bifida, with exceedingly low accuracy for FAScrea. Of note, both BTP-based eGFR equations also performed less accurately in this group compared to all other diagnoses and share a negative bias. There were no significant differences in performance between the eGFR

Table 4: Performance of $eGFR_{BTP,}$ FAScrea, FAScys and the Benlamri equation at different levels of GFR [ml/min/1.73m^2] in terms of %prediction error presented as median [interquartile range] and P_{30} and P_{10} accuracy in % as well as their ability to correctly identify CKD level in %.

GFR		≥90	60-89	≤59
N		188	94	40
Benlamri	*%prediction error [IQR]*	3.1 [-10.7 to 18.1]	14.7 [-0.5 to 37.9]	11.6 [-4.8 to 41.6]
	P30	84.0	73.4	60.0
	P10	37.8	38.3	35.0
	%correctly identified CKD	86.2	43.6	67.5
$eGFR_{BTP}$	*%prediction error [IQR]*	-3.0 [-16.0 to 12.7]	7.3 [-7.7 to 28.7]	-1.7 [-16.5 to 30.3]
	P30	85.1	73.4	65.0
	P10	33.0	38.3	27.5
	%correctly identified CKD	79.3	53.1	70.0
FAScys	*%prediction error [IQR]*	-11.8 [-22.0 to -0.2]	1.6 [-9.6 to 11.3]	9.4 [-3.0 to 25.8]
	P30	86.7	88.3	80.0
	P10	37.8	51.1	30.0
	%correctly identified CKD	64.9	69.1	82.5
FAScrea	*%prediction error [IQR]*	1.0 [-12.1 to 16.3]	10.0 [-1.3 to 21.8]	11.8
	P30	82.5	83.0	65.0 [-12.1 to 33.2]
	P10	36.7	38.3	20.0
	%correctly identified CKD	80.9	53.2	70.0

equations in the other diagnosis groups. Prediction was most accurate in patients with a single kidney.

Considering the effect of glucocorticosteroids (Table 6), we observed a decrease in accuracy but similar %precision error with FAScrea, while both $eGFR_{BTP}$, and FAScys performed less accurately and shifted towards a negative bias in patients on GCS therapy.
When examining differences between males and females, both BTP equations showed a clear decrease in accuracy in adolescent (12 years or older) females, while FAScrea showed a decrease in accuracy for females in younger children (Table 7).

Table 5: Performance of $eGFR_{BTP}$, FAScrea, FAScys and the Benlamri equation in different diagnosis groups in terms of %prediction error presented as median [interquartile range] and P_{30} and P_{10} accuracy in %.

Diagnosis		Malignancy	Nephritis	Single kidney	Spina bifida	Follow up after malignancy	Urological	Other
N		63	45	92	29	13	35	45
Benlamri	*%prediction error [IQR]*	2.3 [-11.8 to 16.1]	2.0 [-12.1 to 18.8]	9.0 [-3.8 to 25.2]	28.5 [2.6 to 51.5]	8.3 [-1.0 to 31.4]	-0.1 [-5.8 to 16.7]	8.5 [-5.1 to 23.2]
	P30	81.0	75.6	82.6	55.2	76.9	77.1	82.2
	P10	36.5	42.2	39.1	17.2	46.2	51.4	31.1
$eGFR_{BTP}$	*%prediction error [IQR]*	-3.7 [-17.3 to 10.3]	-4.5 [-19.1 to 13.2]	2.5 [-9.5 to 18.5]	20.9 [-3.7 to 45.9]	-0.0 [-7.6 to 24.5]	-8.2 [-16.2 to 10.3]	0.9 [-12.5 to 17.0]
	P30	82.5	80.0	84.8	62.1	76.9	77.1	75.6
	P10	36.5	31.1	37.0	24.1	53.9	28.6	31.1
FAScys	*%prediction error [IQR]*	-13.2 [-24.5 to 1.9]	-15.0 [-24.0 to 0.8]	-3.1 [-13.3 to 6.1]	1.0 [-12.4 to 17.5]	1.0 [-5.4 to 9.3]	-3.0 [-16.5 to 4.0]	-2.1 [-16.3 to 11.2]
	P30	77.8	77.8	93.5	89.7	92.3	88.6	86.7
	P10	34.9	24.4	53.3	44.8	61.5	34.3	35.6
FAScrea	*%prediction error [IQR]*	6.3 [-10.7 to 28.7]	3.7 [-12.2 to 18.9]	1.9 [-9.9 to 17.5]	40.9 [10.8 to 90.8]	7.6 [-3.0 to 34.1]	-3.1 [-14.3 to 8.9]	6.4 [-7.4 to 16.4]
	P30	74.6	82.2	89.1	44.8	69.2	94.3	84.4
	P10	34.9	28.9	39.1	13.8	46.2	42.9	37.8

Table 6: Performance of $eGFR_{BTP,}$ FAScrea, FAScys and the Benlamri equation split by glucocorticosteroid use in terms of %prediction error presented as median [interquartile range] and P_{30} and P_{10} accuracy in %

Steroids		Yes	No
N		37	285
Benlamri	*% prediction error [IQR]*	0.7 [-12.9 to 22.6]	7.5 [-5.9 to 25.1]
	P_{30}	70.3	79.0
	P_{10}	37.8	37.5
$eGFR_{BTP}$	*% prediction error [IQR]*	-4.5 [-18.0 to 14.8]	0.2 [-12.0 to 18.2]
	P_{30}	75.7	79.7
	P_{10}	27.0	34.7
FAScys	*% prediction error [IQR]*	-23.5 [-30.8 to -12.3]	-3.1 [-15.5 to 7.0]
	P_{30}	70.3	88.4
	P_{10}	13.5	44.2
FAScrea	*% prediction error [IQR]*	3.7 [-12.8 to 23.9]	4.8 [-9.1 to 20.6]
	P_{30}	73.0	81.4
	P_{10}	27.0	36.1

Table 7: Performance of $eGFR_{BTP,}$ FAScrea, FAScys and the Benlamri equation split by age and sex in terms of %prediction error presented as median [interquartile range] and P_{30} and P_{10} accuracy in %.

Age		Children		Adolecents	
Sex		Male	Female	Male	Female
N		70	60	120	72
Benlamri	*% prediction error [IQR]*	-0.3 [-12.3 to 15.7]	8.7 [-5.3 to 26.7]	4.6 [-6.5 to 19.6]	22.0 [1.2 to 39.2]
	P_{30}	81.4	80.0	80.8	68.1
	P_{10}	35.7	35.0	42.5	33.3
$eGFR_{BTP}$	*% prediction error [IQR]*	-7.0 [-18.5 to 9.3]	2.3 [-11.5 to 20.4]	-1.9 [-13.4 to 12.6]	15.0 [-5.2 to 31.2]
	P_{30}	82.9	80.0	81.7	70.8
	P_{10}	34.3	33.3	39.2	25.0
FAScys	*% prediction error [IQR]*	-6.1 [-19.9 to 2.1]	-2.5 [-12.0 to 6.8]	-10.9 [-19.8 to 2.0]	2.5 [-13.2 to 16.5]
	P_{30}	87.1	83.3	90.8	80.6
	P_{10}	41.3	55.0	36.7	34.7
FAScrea	*% prediction error [IQR]*	1.1 [-14.0 to 16.5]	9.1 [-8.9 to 35.7]	3.9 [-9.0 to 18.6]	9.0 [-7.9 to 23.2]
	P_{30}	85.7	66.7	82.5	83.3
	P_{10}	38.6	28.3	39.2	30.6

Discussion

This study demonstrates that rescaled BTP can be used for GFR estimation in children, using the same principle as described for creatinine (19) and cystatin C (22). It is in line with a recent study using this approach with BTP in elderly patients (27). The resulting equation performs similarly to the creatinine-based FAScrea and the BTP-based Benlamri equation. As in the adult study, addition of more markers enhanced the performance of

the equations, albeit to a lesser extent. It is therefore a matter of debate whether the slight increase in accuracy is worth the extra cost of measuring a third marker. However, since the equations are based on rescaled markers, there is great potential for combining or interchanging different markers depending on specific patient characteristics.

The poorer performance of both cystatin C and BTP in patients on GCS treatment is in line with earlier reports (31, 32), and is most marked for FAScys. This is remarkable as GCS had a stronger effect on BTP in our previous study (32), where BTP concentrations were inversely correlated with GCS dose. Bias of FAScrea was not affected by GCS treatment, which is consistent with earlier reports (33). The poor performance of creatinine in the group with neural tube defects follows reason as these patients are known to have diminished muscle mass. Both $eGFR_{BTP}$ and the Benlamri equation overestimate GFR in spina bifida patients and are less suitable than FAScys in this patient group as described previously (34). The underlying pathophysiology for this observation is unclear.

Analysis of the subgroup with normal GFR revealed a median measured GFR of 100 ml/min/1.73m^2, which is lower than the normal value of 107.3 ml/min/1.73m^2 Pottel used for the calculation of the rescaled eGFR equations (19, 22, 35). This may reflect differences in patient population. In order to conform to his concept and to yield comparable data with FAScys and FAScrea, we used 107.3 ml/min/1.73m^2 in our $eGFR_{BTP}$ equation.
Differences between our population and the population used for the definition of Q_{BTP} probably also account for the lower median GFR in our patients with normal GFR and the patients from the earlier cohort (2), which was used for the definition of Q_{BTP}. In the latter, mean Schwartz-GFR was 108 ml/min/1.73m^2. This may explain why the Q-value of 0.69 mg/l is lower than the median concentration of 0.73 mg/l observed in our subgroup with normal kidney function. Here, too, we chose not to adapt the Q_{BTP} value to the findings in our population in order to avoid a bias favoring the in-house $eGFR_{BTP}$ equation.
We observed significantly higher serum levels of BTP in boys, which have also been reported by a number of other studies (11, 27, 30, 36) but not in our earlier publication and in the study by Filler et al (37). Considering our Table 7, it is quite possible that the difference in gender does not arise until adolescent age, i.e. cohorts with younger children will not report gender related differences, whereas cohorts with older children will. Of note, the Benlamri equation, which was developed in children at median age of 11 years, also does not differentiate for gender.

There have been no reports indicating an association between age or height and BTP concentrations. Still, we observed a trend towards an inverse relation with BTP concentrations and age and height. This may be the reason why Pottel found a lower Q_{BTP}

of 0.60 mg/l in elderly subjects (27). This indicates that a potential BTP equation covering the full age spectrum might require age-specific Q values. Filler et al reported that thyroid function might alter BTP concentrations (37). This was not addressed in the present study.

Our study has several limitations. (i) The Q-value was adapted from a group of children without known kidney disease (2). GFR had not been measured in these children, however. Also, the children were younger (10.4 ± 4.6 years) than the patients in the present study. This may account for the differences in median BTP concentration in the patients with normal kidney function and the Q-value used in our equation. Still, $eGFR_{BTP}$ performs very well in our population and had remarkably low bias. Possibly, the Q-value can be adapted in a larger study, optimally covering the whole age spectrum. It is conceivable that such a study will come up with gender-specific Q-values from adolescence (ii) Our patients were relatively old for a pediatric population. This has to be borne in mind when extrapolating our findings to a general pediatric population. (iii) Unlike for creatinine and cystatin C (38-40) no international reference standard for BTP measurement has been established to date. In the past, differences in assay standardization were a major obstacle for the implementation of cystatin C in clinical practice (37). Therefore, caution is warranted when extrapolating the results from this study to populations where a different assay for BTP measurement is used.

Conclusion

Rescaled BTP concentrations are a simple method for estimating GFR in children. However, in our cohort, they provided little additional benefit over rescaled creatinine and cystatin C. In the future, this method might be used to develop an equation covering the full age spectrum from pediatrics to old age.

Conflicts of interest: None

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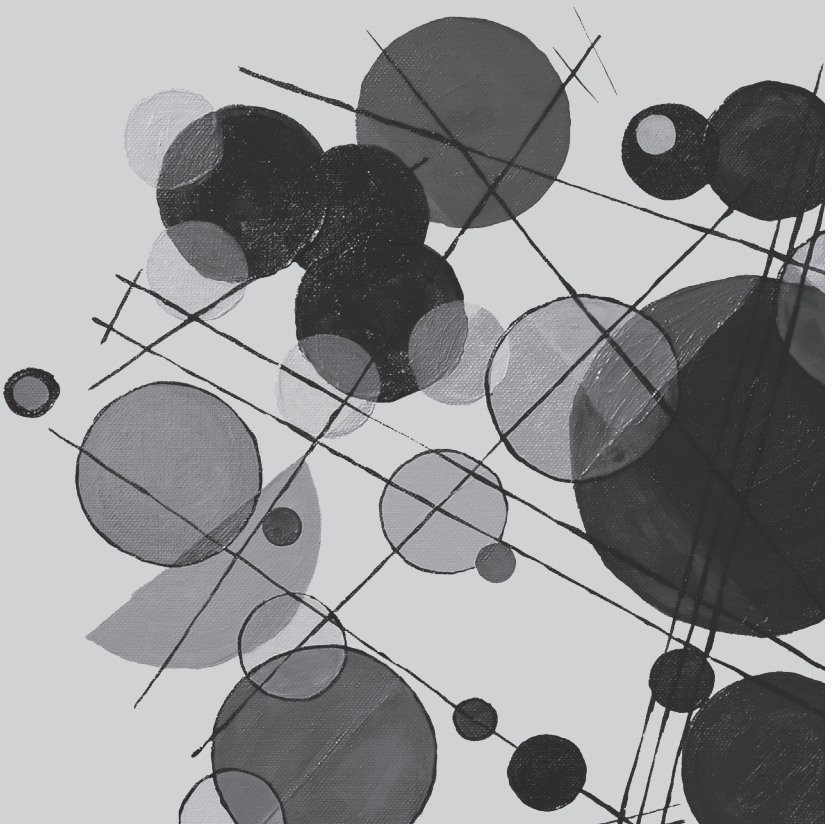

Shrunken Pore Syndrome in children- fact or fiction?

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Abstract

Introduction

The link between cystatin C (cysC) and mortality independently of glomerular filtration rate (GFR) in adults has prompted the "*Shrunken Pore Syndrome*" (SPS) hypothesis, where the constellation of high serum cysC with normal creatinine is explained by reduced glomerular pore size, through which creatinine can pass freely, while the larger cysC, beta-trace protein (BTP) and pro-inflammatory molecules are retained.

This study set out to apply the definition of SPS to children also using a gold standard GFR measurement.

Methods

In 294 children who underwent a inulin clearance (Cin) test serum creatinine, cysC and BTP were measured. For all three markers $eGFR_x$ was calculated using the full age spectrum equations. The ratio $eGFR_{cys}/eGFR_{crea}$ was plotted against the %prediction error (($eGFR_x$-Cin)/Cin*100%) of $eGFR_{BTP}$. Patients with and without SPS according to different cut-off points of $eGFR_{cys}/eGFR_{crea}$ and $eGFR_{cys}$/Cin (i.e. ≤0.6,0.7,0.8) were compared in terms of $eGFR_x$, Cin, %prediction error and $eGFR_{BTP}/eGFR_{crea}$-ratio.

Results

A positive correlation between $eGFR_{cys}/eGFR_{crea}$ and %prediction error for $eGFR_{BTP}$ was found. Patients with SPS had lower %prediction error for $eGFR_{cys}$ and $eGFR_{BTP}$ and higher Cin regardless of the definition. However, the overestimation of $eGFR_{crea}$ in patients with SPS was only present when using the $eGFR_{cys}/eGFR_{crea}$ rather than the $eGFR_{cys}$/Cin definition challenging the causality between shrunken pores and muscle wasting.

Conclusion

CysC and BTP are related independently of creatinine, suggesting glomerular pore size as a common denominator. For research in SPS, the definition should be based on $eGFR_{cys}$/Cin rather than the $eGFR_{cys}/eGFR_{crea}$–ratio to exclude extrarenal factors affecting creatinine metabolism.

Introduction

Cystatin C, a low molecular weight protein marker of GFR, has been correlated to increased all-cause and cardiovascular mortality, independently of kidney function.(1-4) When comparing the creatinine-based with the cystatin C- based estimated GFR ($eGFR_{crea}$ and $eGFR_{cys,}$ respectively) in adults, Grubb et al noted that in around 8% of their adult population, $eGFR_{cys}$ was less than 60% of the respective $eGFR_{crea}$.(5) This group had excess mortality, both in the subgroups with advanced renal failure (CKD stage 3 and higher) and in CKD 1 and 2 patients.(6) Beta-trace protein (molecular weight (MW) 23 kDa) and beta-2 microglobulin (MW 11.8 kDa), two other low-molecular weight protein (LMWP) markers of GFR along with renally excreted macromolecules also accumulate in excess of creatinine in this subgroup.(7, 8) During the course of the pregnancy cystatin C, along with beta-trace protein and beta-2 microglobulin concentrations were shown increase relative to measured GFR, while no placental production was found, suggesting alterations in filtration selectivity.(9, 10)

Furthermore in pregnant women elevated cystatin C, beta-trace protein and beta-2 microglobulin concentrations with normal levels of creatinine have been associated with an increased incidence of pre-eclampsia.(11)

These observations prompted Grubb et al to propose the concept of a "*Shrunken Pore Syndrome*", i.e. an alteration in glomerular pore size underlying the discordance between the LMWP GFR markers and creatinine associated with adverse outcomes. In *Shrunken Pore Syndrome* cystatin C (MW 13.3 kDa) accumulates in excess of creatinine (MW 113 Da) due to changes in the filtration characteristics of the glomerular filter. The alterations in glomerular pore size lead to accumulation not only of LMWP GFR-markers, but also of pro-inflammatory molecules of similar size such as interleukin 1-β (MW 17kDa) and interleukin 6 (MW 23-25 kDa).(12, 13) This might lead to a chronic inflammatory state underlying the observed excess mortality in patients with *Shrunken Pore Syndrome*. In support of this concept, Almen et al (37) demonstrated size-specific accumulation of molecules associated with artherosclosis in patients with *Shrunken Pore Syndrome*.

In line with observations in adults, we recently described a similar distribution of $eGFR_{cys}$ compared to $eGFR_{crea}$ in children where larger discrepancies between the two reflected overestimation of $eGFR_{crea}$ and underestimation of $eGFR_{cys}$.(14) We therefore hypothesize that the changes in filtration characteristics observed in *Shrunken Pore Syndrome* are present in children, too. Aim of the present study was to compare the two LMWP GFR markers cystatin C and beta-trace protein with serum creatinine using inulin clearance as gold standard to establish a physiological link between cystatin C and beta-trace protein independent of the filtration of small solutes like creatinine or inulin, which would support the existence of *Shrunken Pore Syndrome* in children.

Methods

Study population

We retrospectively analyzed data on pediatric patients who underwent a single injection inulin clearance test on clinical grounds between 2004 and 2015. We excluded patients with neural tube defects, since the body composition of these patients precludes effective use of both creatinine and beta-trace protein as markers for kidney function (15) as well as patients undergoing glucocorticosteroid treatment since this is known to affect both cystatin C and beta-trace protein concentrations.(16) Also, patients with thyroid dysfunction were excluded.(17)

Analytical methods

The single injection inulin clearance (Cin) was performed as described previously.(18, 19) In short, patients were injected with a single dose of inulin (Inutest®, Fresenius, Bad Homburg, Germany) of 5000 mg/m^2, with a maximum of 5000 mg. Subsequently at timed intervals blood was taken for inulin measurement which was done using an enzymatic method. GFR was calculated from the rate of decline of inulin concentrations. During the test, blood was taken for the measurement of creatinine, cystatin C and beta-trace protein. From 2008 onwards, creatinine was measured using the creatinase-sarcosine oxidase enzymatic method, which is traceable to isotope dilution mass spectrometry (IDMS), before 2008 a kinetic Jaffe method was used. The low-molecular weight proteins were measured by particle-enhanced immunonephelometry on a Behring Nephelometer II (Siemens Healthcare, Marburg, Germany), which in the case of cystatin C was calibrated to the International Federation of Clinical Chemists (IFCC) standard.(20) For both creatinine and cystatin C data pre-dating IFCC standardization were recalculated using the factors described in an earlier publication (IFCC creatinine in µmol/l= non-IFCC creatinine in µmol/l × 1.1- 26; IFCC cystatin C in mg/l = non-IFCC cystatin C in mg/l x 1.17).(21)

eGFR equations

In order to minimize bias from equation structure, we selected eGFR equations that were established following the same principle and which have been demonstrated to perform well in our hands.(14, 15, 21) These equations are:

(i) The creatinine-based FASage equation (22)
$eGFR_{crea}$ [ml/min/1.73 m^2] = 107.3/creatinine/Q_{crea}
with creatinine in mg/dl and Q_{crea} being the age-appropriate median creatinine concentration in healthy subjects.

(ii) The cystatin C-based FAScys equation (23)
$eGFR_{cys}$ [ml/min/1.73 m^2] = 107.3/cystatin C/0.82
with cystatin C in mg/l

(iii) The beta-trace protein-based FAS_{BTP} equation (15)
$eGFR_{BTP}$ [ml/min/1.73 m^2] = 107.3/beta-trace protein/0.69
with beta-trace protein in mg/l

Definitions
To assess the accuracy of the equations within this population we use P_{10} and P_{30} accuracy levels, which describes the percentage of cases in which $eGFR_x$ is within ±10 or ±30% of Cin, respectively.

The common definition of *Shrunken Pore Syndrome* is $eGFR_{cys}/eGFR_{crea} \leq 0.6$ (6), which is also applied in this study. Besides, an inulin clearance-based definition is used, i.e. $eGFR_{cys}$/Cin below arbitrary thresholds of 0.6, 0.7 and 0.8.

To identify whether there is over- or underestimation of $eGFR_x$ compared to the gold standard we use %prediction error, defined as

(i) $(eGFR_x\text{-Cin})/Cin \times 100\%$

The Cin-based definition of *Shrunken Pore Syndrome* is mathematically linked to %prediction error: $eGFR_{cys} \leq 0.6$ Cin corresponds to a %prediction error for $eGFR_{cys}$ of -40% or lower.

Statistical methods
The relation between $eGFR_{BTP}$ and $eGFR_{cys}/eGFR_{crea}$ is analyzed using linear regression analysis with the ratio as dependent variable and the %prediction error of beta-trace protein as independent variable. Results are given as B-values with 95% confidence intervals (CI), statistical significance is defined as $p<0.05$.
Differences in eGFR, Cin, %prediction error and $eGFR_{BTP}/eGFR_{crea}$ ratio between groups of patients who fit the definition of *Shrunken Pore Syndrome* and those who do not are given in medians [interquartile range (IQR)] and statistical significance is tested by independent samples median test. Here a non-parametric test is used because of the size differences between the groups, causing a non-Gaussian distribution. All statistical analyses were performed using SPSS version 25.

Results

Patient characteristics and performance of eGFR equations

Data on 294 unique patients undergoing inulin clearance tests were analyzed. Mean age was 12.6 years (range 2.1-19.5), mean Cin was 90.9 ml/min/1.73m^2 (range: 13.4-185.0). 59.2% of patients were male. The primary diagnosis was malignancy in 20.7% of patients, single kidney in 31.6%, nephritis in 15.3%, urological in 12.2%, follow-up after malignancy in 4.4% and other in 15.6%.

The performance of all three eGFR equations in our population in terms of mean %prediction error and P_{30} and P_{10} accuracy rates is summarized in Table 1, with all three equations performing adequately.

Table 1: Performance of the three equations

	%prediction error (SD)	P_{30} accuracy	P_{10} accuracy
$eGFR_{crea}$	9.3 (39.1)	83.7	37.1
$eGFR_{cys}$	-4.7 (20.1)	85.7	40.1
$eGFR_{BTP}$	1.0 (27.4)	82.7	36.7

Relationship between beta-trace protein and the $eGFR_{cys}/eGFR_{crea}$ ratio

The %prediction error of beta-trace protein is plotted against the ratio $eGFR_{cys}/eGFR_{crea}$ in Figure 1. Here, a positive correlation is seen between %prediction error of beta-trace

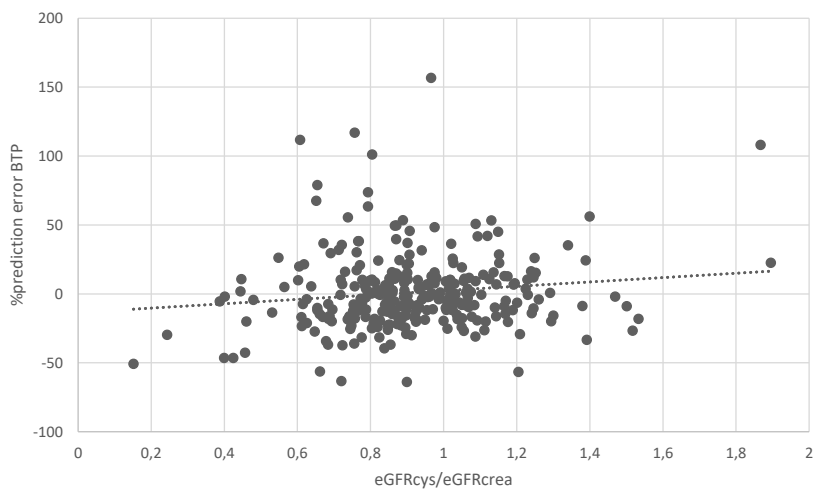

Figure 1: Relationship between the ratio $eGFR_{cys}/eGFR_{crea}$ and %prediction error of beta-trace protein

protein and the $eGFR_{cys}/eGFR_{crea}$ ratio, indicating underestimation of GFR by beta-trace protein with decreasing $eGFR_{cys}/eGFR_{crea}$ (B=0.107 [95% CI 0.202 to 0.013], p=0.026).

Shrunken Pore Syndrome defined by the $eGFR_{cys}$ /$eGFR_{crea}$ ratio

Grubb et al suggest the somewhat arbitrary cut-off point of $eGFR_{cys} \leq 0.6\ eGFR_{crea}$ for the definition of *Shrunken Pore Syndrome*.(5) However, other cut-off points have been used, too.(8) Table 2 compares patients who fit the definition for *Shrunken Pore Syndrome* (SPS+) and those who do not (SPS-) using cut-off points of 0.6 and 0.7. Depending on the cut-off used the prevalence is 4.8% and 13.9%.

Table 2: Comparison between patients with (SPS+) and without (SPS-) Shrunken Pore Syndrome defined by different cut-offs of $eGFR_{cys}$ /$eGFR_{crea}$.
Data presented as median values [IQR], statistical analysis using independent samples median-test.

	$eGFR_{cys}$ / $eGFR_{crea} \leq 0.6$		
	SPS+ (n=14)	SPS- (n=280)	p
$eGFR_{cys}$ [ml/min/1.73m²]	85.5 [53.8 to 94.3]	85.9 [70.9 to 97.7]	0.784
$eGFR_{BTP}$ [ml/min/1.73m²]	89.1 [64.0 to 111.7]	90.8 [71.1 to 107.9]	0.804
$eGFR_{crea}$ [ml/min/1.73m²]	182.8 [126.7 to 216.7]	92.5 [75.2 to 109.7]	0.003
Cin [ml/min/1.73m²]	109.6 [95.0 to 131.6]	92.2 [74.5 to 107.3]	0.055
%prediction error cystatin C	-32.5 [-47.3 to -13.4]	-4.2 [-17.0 to 5.2]	0.014
%prediction error beta-trace protein	-9.5 [-43.6 to 2.7]	-2.5 [-15.0 to 10.9]	0.411
%prediction error creatinine	46.9 [25.8 to 112.6]	2.9 [-11.0 to 14.9]	0.000
$eGFR_{BTP}/eGFR_{crea}$	0.45 [0.40 to 71.5]	0.99 [0.83 to 1.12]	0.003
	$eGFR_{cys}$ / $eGFR_{crea} \leq 0.7$		
	SPS+ (n=41)	SPS- (n=253)	p
$eGFR_{cys}$ [ml/min/1.73m²]	86.4 [66.3 to 95.2]	85.5 [71.3 to 107.9]	1.000
$eGFR_{BTP}$ [ml/min/1.73m²]	90.8 [65.9 to 110.9]	90.8 [71.3 to 107.9]	0.963
$eGFR_{crea}$ [ml/min/1.73m²]	136.9 [110.1 to 176.9]	89.9 [74.2 to 105.8]	0.000
Cin [ml/min/1.73m²]	103.0 [89.4 to 119.0]	91.4 [73.1 to 106.9]	0.007
%prediction error cystatin C	-22.7 [-32.9 to -9.3]	-3.4 [-15.8 to 6.1]	0.000
%prediction error beta-trace protein	-11.4 [-22.2 to 7.7]	-1.3 [-13.0 to 10.8]	0.043
%prediction error creatinine	28.6 [13.9 to 63.6]	0.7 [-12.0 to 12.5]	0.000
$eGFR_{BTP}/eGFR_{crea}$	0.68 [0.45 to 0.84]	1.00 [0.86 to 1.12]	0.000

Patients who fit the definition of *Shrunken Pore Syndrome* have significantly lower %prediction error for cystatin C and higher %prediction error for creatinine, regardless

of the cut-off level used. This is to be expected since this group is defined by low $eGFR_{cys}$ compared to $eGFR_{crea}$ and therefore underestimation of GFR by $eGFR_{cys}$ and overestimation by $eGFR_{crea}$ is likely. Interestingly the difference in $eGFR_{cys}$ is very small, indicating that the difference in %prediction error is mainly due to shifts in Cin between the two groups as Cin levels are higher in SPS+ than SPS-. The link between $eGFR_{cys}$ / $eGFR_{crea}$ and Cin was confirmed by linear regression analysis demonstrating a significant negative correlation (B = -0.228 [95% CI -0.313 to -0.143], p=0.000). Like $eGFR_{cys}$, $eGFR_{BTP}$ is very similar between SPS+ and SPS-, while %prediction error for beta-trace protein tends to be more negative in the SPS+ group reaching significance when a cut-off of 0.7 is used. Also, $eGFR_{BTP}$ / $eGFR_{crea}$ is significantly lower in SPS+ patients irrespective of the cut-off used to define *Shrunken Pore Syndrome*.

When comparing cystatin C and creatinine, the changes for creatinine are more pronounced than for cystatin C indicating that the $eGFR_{cys}$ / $eGFR_{crea}$–based definition is strongly driven by creatinine. This is a potential confounding factor in *Shrunken Pore Syndrome* research as patients with adequate levels of cystatin C for their GFR but abnormally low levels of creatinine for any reason also meet the definition of *Shrunken Pore Syndrome*. To counter this problem, we studied a new definition based on $eGFR_{cys}$/Cin.

***Shrunken Pore Syndrome* defined by the $eGFR_{cys}$ /Cin ratio**

Table 3 compares patients who fit the definition for *Shrunken Pore Syndrome* (SPS+) and those who do not (SPS-) using cut-off points of $eGFR_{cys}$ /Cin of 0.6, 0.7 and 0.8. Depending on the cut-off used the incidence of *Shrunken Pore Syndrome* is 2.7%, 8.2% and 20.4%.

Here, too, the lower %prediction error for cystatin C in the SPS+ group is driven by a higher Cin rather than a lower $eGFR_{cys}$. The %prediction error of both low-molecular weight protein markers is lower in the SPS+ group indicating impaired filtration while there is little to no difference for creatinine. While the $eGFR_{BTP}$/$eGFR_{crea}$ – ratio was statistically lower in SPS+ patients using the $eGFR_{cys}$ / $eGFR_{crea}$-based definition this is not the case when using the inulin clearance-based definition.

Discussion

This is the first study addressing *Shrunken Pore Syndrome* by studying the relationship between the low-molecular weight protein markers cystatin C and beta-trace protein versus serum creatinine in children using inulin clearance as golden standard. Our main findings are that cystatin C and beta-trace protein are linked independently from serum creatinine and that the definition of *Shrunken Pore Syndrome* based on $eGFR_{cys}$ /Cin rather than the commonly used definition by $eGFR_{cys}$ / $eGFR_{crea}$ abolishes the relation with

Table 3: Comparison between patients with (SPS+) and without (SPS-) Shrunken Pore Syndrome defined by different cut-offs of $eGFR_{cys}$ /Cin
Data presented as median values [IQR], statistical analysis using independent samples median-test.

	$eGFR_{cys}$ / Cin ≤ 0.6		
	SPS+ (n=8)	SPS- (n=286)	p
$eGFR_{cys}$ [ml/min/1.73m²]	79.5 [50.9 to 96.2]	86.3 [70.9 to 97.7]	0.720
$eGFR_{BTP}$ [ml/min/1.73m²]	110.1 [80.8 to 138.0]	90.0 [70.2 to 107.9]	0.274
$eGFR_{crea}$ [ml/min/1.73m²]	130.7 [112.0 to 205.1]	93.0 [75.3 to 110.8]	0.073
Cin [ml/min/1.73m²]	152.7 [100.7 to 171.2]	92.3 [74.6 to 107.4]	0.073
%prediction error cystatin C	-45.4 [-50.5 to -41.3]	-4.4 [-17.1 to 5.4]	0.012
%prediction error beta-trace protein	-22.1 [-42.2 to -2.9]	-2.5 [-14.7 to 10.7]	0.282
%prediction error creatinine	22.8 [-19.2 to 39.0]	3.5 [-10.4 to 16.5]	0.282
$eGFR_{BTP}/eGFR_{crea}$	0.73 [0.43 to 1.05]	0.97 [0.82 to 1.11]	0.720
	$eGFR_{cys}$ / Cin ≤ 0.7		
	SPS+ (n=24)	SPS- (n=270)	p
$eGFR_{cys}$ [ml/min/1.73m²]	84.0 [67.2 to 91.4]	86.3 [70.9 to 97.7]	0.831
$eGFR_{BTP}$ [ml/min/1.73m²]	105.7 [77.6 to 129.0]	89.7 [69.7 to 107.8]	0.271
$eGFR_{crea}$ [ml/min/1.73m²]	116.2 [98.3 to 130.9]	92.1 [74.9 to 109.7]	0.006
Cin [ml/min/1.73m²]	127.9 [102.0 to 149.8]	91.4 [72.7 to 105.9]	0.000
%prediction error cystatin C	-34.8 [-41.7 to -33.0]	-3.3 [-15.7 to 6.2]	0.000
%prediction error beta-trace protein	-19.2 [-31.0 to -9.9]	-1.0 [-13.0 to 11.2]	0.000
%prediction error creatinine	-6.6 [-23.6 to 19.2]	4.1 [-9.3 to 17.3]	0.287
$eGFR_{BTP}/eGFR_{crea}$	0.95 [0.69 to 1.10]	0.97 [0.82 to 1.11]	0.831
	$eGFR_{cys}$ / Cin ≤ 0.8		
	SPS+ (n=60)	SPS- (n=234)	p
$eGFR_{cys}$ [ml/min/1.73m²]	83.1 [70.3 to 90.6]	86.4 [70.9 to 100.3]	0.311
$eGFR_{BTP}$ [ml/min/1.73m²]	91.9 [75.4 to 114.1]	90.5 [69.5 to 107.8]	0.839
$eGFR_{crea}$ [ml/min/1.73m²]	110.0 [88.2 to 129.1]	90.3 [72.8 to 107.4]	0.006
Cin [ml/min/1.73m²]	112.6 [95.6 to 128.5]	89.1 [68.5 to 102.0]	0.000
%prediction error cystatin C	-26.5 [-33.9 to -23.3]	-0.3 [-11.5 to -8.4]	0.000
%prediction error beta-trace protein	-16.9 [-24.8 to -7.5]	0.8 [-10.7 to 13.2]	0.000
%prediction error creatinine	-4.8 [-17.3 to 12.1]	4.8 [-6.1 to 17.6]	0.030
$eGFR_{BTP}/eGFR_{crea}$	0.90 [0.72 to 1.09]	0.99 [0.83 to 1.12]	0.111

diminished serum creatinine challenging the direct link between shrunken pores and muscle wasting.

The similar distribution of the two LMWP markers can potentially be explained by a number of renal and non-renal factors: [1] The rate of synthesis might be linked, [2] there might be similarities in renal tubular re-absorption or [3] the equations used to estimate eGFR might add a bias depending on the population where they were derived and [4] glomerular elimination might be linked by either similar electric charge or similar molecular size. Of course, all these potential similarities must not apply to creatinine and measured GFR in order to fit with our findings.

It has been shown that production sites and production pathways of all three markers are different. (24, 25) However, several factors influencing the production of beta-trace protein and cystatin C such as genetic polymorphisms (26, 27) as well as age and gender differences (28, 29) are still being investigated so that some links in the synthesis of the two LMWP markers might still emerge. With regards to renal tubular handling, there are some differences between the markers: while both cystatin C and beta-trace protein undergo tubular reabsorption and degradation, reabsorption of beta-trace protein is incomplete so that beta-trace protein is found in the urine of healthy individuals.(30) It is also unlikely that our findings are related to the equations used to estimate eGFR as the mean %prediction error for $eGFR_{BTP}$ is positive for the population as a whole so that the negative %prediction error found in patients with *Shrunken Pore Syndrome* defined by $eGFR_{cys}/eGFR_{crea}$ or $eGFR_{cys}$/Cin ratios cannot be explained by bias within the equations. The iso-electric point differs being 9.3 for cystatin C and 5.8-6.7 for beta-trace protein.(31)

Therefore, the strongest link between cystatin C and beta-trace protein when compared to creatinine and inulin is molecular weight, which is 23-29 kDa for beta-trace protein and 13.3 kDa for cystatin C, while the molecular weight of inulin is 5 kDa and 0.113 kDa for creatinine (31). Although the glomerular sieving of beta-trace protein (30) is less well defined than cystatin C, (32) molecular size affecting passage through the endothelial pores is the most likely common denominator.(33) Therefore changes in glomerular sieving characteristics as observed during pregnancy and in particular in pre-eclampsia lead to a parallel rise in cystatin C, beta-trace protein and other molecules of similar size, (34) an observation which led Anders Grubb to coin the term "*Shrunken Pore Syndrome*". (5)

While one would expect $eGFR_{cys}$ or $eGFR_{BTP}$ to be decreased in SPS+ patients this is not the case. Still, %prediction errors of both parameters are strongly negative in SPS+ patients indicating underestimation in relation to a much higher Cin. This finding is in line with physiology data describing an inverse relationship between glomerular sieving coefficients and GFR for molecules the size of cystatin C and beta-trace protein while

the sieving coefficient of small molecules like creatinine is not affected by GFR.(35, 36) This implies that these markers are not suitable for the recognition of hyperfiltration in diabetics as demonstrated by Perrin and Berg.(37) The study by Huang et al also showed an inverse relationship between measured GFR and $eGFR_{cys}$ while the opposite was true for $eGFR_{crea}$ and $eGFR_{BTP}$ was the least affected.(38)

The most common definition of *Shrunken Pore Syndrome* relies on the relationship between cystatin C and creatinine (8) or the $eGFR_{cys}/eGFR_{crea}$ ratio.(6) Due to the age-dependency of creatinine production only the latter is applicable in children (39). Using this definition, a low $eGFR_{cys}$ is associated with a high $eGFR_{crea}$ reflecting accumulation of cystatin C and a decrease in serum creatinine (5, 14). One potential explanation for this is the accumulation of pro-inflammatory molecules, which are roughly the same size as the LMW proteins. This theory is supported by a recent study using proteomics, which showed higher concentrations of atherosclerosis promoting proteins in patients with *Shrunken Pore Syndrome* compared to GFR-matched controls.(40) This study also demonstrated an inverse relationship between molecular size and accumulation in *Shrunken Pore Syndrome*, a trend which can also been seen in our data where underestimation of GFR by cystatin C is more pronounced than by beta-trace protein.

The Shrunken Pore concept proposes that accumulation of pro-inflammatory molecules induces a catabolic state with muscle wasting and decreased creatinine production leading to overestimation $eGFR_{crea}$. This catabolic state may also explain the increased mortality risk associated with cystatin C in general (1-4) and in patients with *Shrunken Pore Syndrome*, in particular.(6, 41) Indeed, there is a similar link between beta-trace protein and beta-2 microglobulin and cardiovascular and all-cause mortality.(42-45) It must be borne in mind, however, that in the definition used to identify patients with *Shrunken Pore Syndrome* in all these studies is strongly affected by serum creatinine levels or $eGFR_{crea}$.

When comparing tables 2 and 3 the strongly positive %prediction error for $eGFR_{crea}$ in SPS+ patients defined by $eGFR_{cys}/eGFR_{crea}$ disappears when Shrunken Pores are defined based on Cin. This challenges the pathophysiological concept described above and suggests that in many patients extra-renal factors underlie the low $eGFR_{cys}/eGFR_{crea}$ ratio and may confound the findings in mortality and morbidity rates as well as the higher levels of atherosclerosis found in these patients.(40) This is the reason why we excluded patients with known interference with the markers studied, e.g. neural tube defects, anorexia nervosa and steroid use (16, 25) and propose a more stringent definition of *Shrunken Pore Syndrome* based on Cin. Although this definition is not applicable in clinical practice where gold standard GFR measurements are rarely performed it should be tested in research

settings. Several recent studies linking clinical outcome data with measured GFR, cystatin C and beta-trace protein (44-46) may be useful to determine the cut-off for $eGFR_{cys}$/Cin to define *Shrunken Pore Syndrome*. It is important in this regard to realize that besides potential differences in pore size there are many renal and non-renal factors involved as well as unavoidable collinearity with Cin as described by Tangri et al.(44)

The association between the accumulation of LMWP markers and mortality found in adult populations is a gradual effect and does not appear to be an "on-off" phenomenon. (1) This is reflected by the decreasing %prediction errors when broadening the definition of *Shrunken Pore Syndrome* in Table 2 and 3. Therefore, the cut-off point suggested to define *Shrunken Pore Syndrome* is somewhat arbitrary and also affected by the eGFR equations used.(8) Grubb et al suggest a cut-off point of $eGFR_{cys}/eGFR_{crea} \leq 0.6$, which is a level at which a clear difference in mortality is seen in adults, giving *Shrunken Pore Syndrome* its clinical relevance.(6) Using this cut-off in our population we find a prevalence of 4.8% which is in line with reported prevalence in adults ranging between 2.1% and 5.7% depending on the eGFR equation and population studied.(6) However, both much lower (0.2-0.7%) (8) and higher (10.5-22.1%) (41) prevalence has been reported, too. With the Cin-based definition, the prevalence dropped to 2.7% when using a cut-off of 0.6. It might therefore be preferable to use a higher cut-off of 0.7 (prevalence 8.2%) yielding higher statistical power when comparing SPS+ and SPS- patients. Still, ultimately, the cut-off to define *Shrunken Pore Syndrome* needs to be defined based on hard clinical end-points (e.g. pre-eclampsia, mortality) using ROC analysis.

Our study has a number of limitations. First, as noted above, the definition of *Shrunken Pore Syndrome* should be based on adverse mortality outcomes, which is not feasible in a pediatric population. Second, the cross-sectional set-up of our study precluded an analysis of the intrapersonal variation of eGFRcys/Cin. Based on the association with adverse long-term outcomes one would expect that the presence of Shrunken Pores is a patient characteristic with little variability, in particular if defined based on Cin and not on $eGFR_{crea}$. It is also conceivable, however, that glomerular filtration characteristics in children and adolescents change with aging calling for serial measurements. Third, although in line with adult data, the prevalence data described need further study as they are currently based on arbitrary cut-offs.

Conclusion

Cystatin C and beta-trace protein are related independently of creatinine in children, too, suggesting glomerular pore size as a common denominator. For research in *Shrunken Pore Syndrome*, the definition should be based on $eGFR_{cys}$/Cin rather than the $eGFR_{cys}/eGFR_{crea}$ – ratio in order to exclude extrarenal factors affecting creatinine metabolism.

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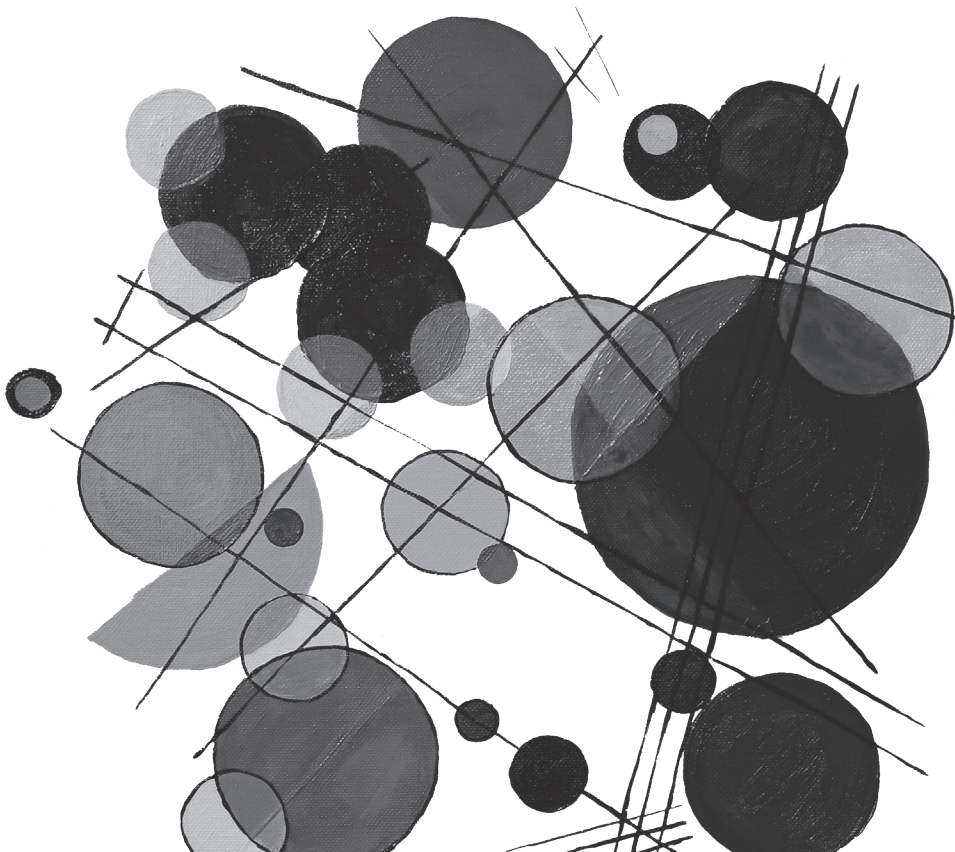
Section two

Combining markers



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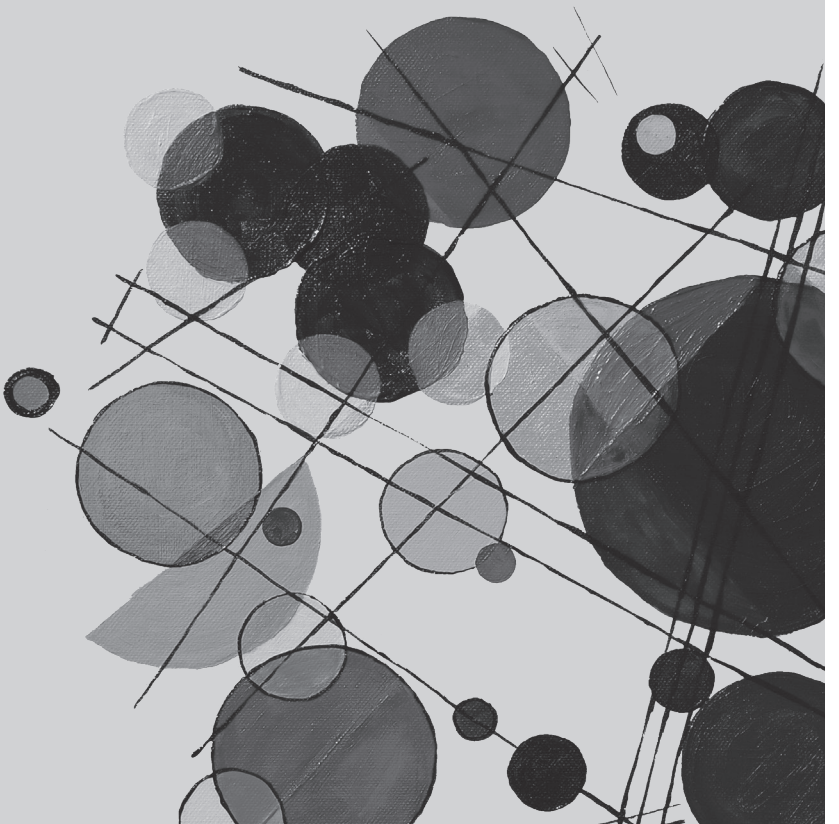

Accurate eGFR reporting for children without anthropometric data

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Introduction

Current guidelines advise reporting estimated GFR (eGFR) instead of serum creatinine concentrations as there is evidence that this leads to earlier recognition and referral of adult patients with kidney failure(1). This is even more important for children, where creatinine production increases with growth leading to changing reference values during childhood and adolescence.(2) As muscle mass is closely related to height in children, the most commonly used equations developed by Schwartz et al, Counahan et al and Gao et al incorporate height (3-6), a patient characteristic not readily available to many laboratories. Height-independent equations based on age-specific normal values in children were developed by Pottel et al (7, 8) and have recently been extended to the full age spectrum above the age of 2 years (9). Since the date of birth is typically used as an identifier in clinical practice, information on age is known to the clinical laboratories which allows direct reporting of eGFR instead of serum creatinine levels using this method. However, the Pottel equation is not sufficiently accurate for general use, with a p30 accuracy of around 75% (i.e. 75% of estimates lie within ±30% of a gold standard measurement) (10), whereas the CKD-EPI equation used by many laboratories to report eGFR in adults has a p30 accuracy of around 85% (11, 12).

In contrast to creatinine, cystatin C production is independent of anthropometric characteristics allowing for direct reporting of a cystatin C-based eGFR by the laboratory. Combining height-dependent creatinine-based equations with those based on cystatin C has been shown to significantly increase accuracy of eGFR both in adults and in children (13-15). The objective of this study is to assess whether combining a *height-independent* creatinine equation with cystatin C-based equations can adequately estimate GFR in children. It is set up as a non-inferiority study against a combination of a *height-dependent* creatinine-based and a cystatin C-based equation.

Methods

Materials

We retrospectively analyzed data in pediatric patients who had undergone an inulin clearance test (Cin) on clinical grounds and in whom creatinine, cystatin C and urea had been measured during the clearance study as part of routine patient care. Cin was measured using a single injection plasma-disappearance technique as published previously (9). In short, patients received a single intravenous dose (5000 mg /1.73m² of body surface area with a maximum dose of 5000 mg) of inulin (Inutest®, Fresenius, Bad Homburg, Germany) within 1 minute. Serial blood samples were obtained at 10, 30, 90 and 240 minutes after

injection. Inulin concentrations were measured in serum by an enzymatic method (16) and inulin clearance in ml/min/1.73m² was calculated from the decline of serum levels, using MW/Pharm 3.5 software (Mediware, Groningen, The Netherlands), a pharmacokinetic computer program using a Bayesian estimate from patient and population data (17).
From 2008 onwards, serum creatinine was measured using the IDMS traceable creatinase/sarcosine oxidase enzymatic method.(18) Before this a kinetic Jaffe method was used, which was subsequently converted to fit the later IDMS traceable method by a conversion equation established locally at the department of clinical chemistry. For cystatin C a particle-enhanced immunonephelometric assay (PENIA; Siemens Healthcare, Marburg, Germany) on a Behring Nephelometer II was used in accordance with the method used by Schwartz et al (3). Measurements performed after IFCC-calibration of the Siemens assay were divided by 1.17 to fit the original calibration of the cystatin C-based Schwartz equations. Urea was measured using an enzymatic method.
Patient characteristics were collected by chart analysis and analyzed in an anonymized data base.

Equations
For the current study we chose eGFR equations, which have been used successfully in our patient population, i.e. the CKiD equations by Schwartz et al (3, 19) and the Pottel equation (10, 14).

(i) $Schwartz_{cys} = 40.6 \times (1.8/\text{cystatin C})^{0.93}$
with cystatin C concentrations in mg/l

For creatinine, the *height-dependent* recalibrated Schwartz (3) equation ($Schwartz_{crea}$) was compared with the *height-independent* FASage equation, which is based on the normal values per age group (9):

(ii) $Schwartz_{crea} = 42.3 \times (\text{ht/creatinine})^{0.79}$
with height in meters and creatinine concentration in mg/dl

(iii) FASage = 107.3 / (creatinine/Q)
with creatinine concentration in mg/dl and Q being the age specific normal value of creatinine

For comparison we used the height-dependent complex CKiD-3 equation, which combines creatinine, cystatin C, urea, gender and anthropometric data (2).

(iv) eGFR-CKiD-3 = 39.8 (ht/creatinine)$^{0.456}$ x (1.8/cystatin C)$^{0.418}$ x (30/urea)$^{0.079}$ x 1.076^{male} x (ht/1.4)$^{0.179}$

with height in meters, creatinine concentration in mg/dl, cystatin C concentration in mg/l, urea concentration in mg/dl and male coded as 1 and female as 0

Statistical analysis

The analytical performance of the equations was characterized by assessing bias, %precision error, absolute %precision error, P_{10} and P_{30} accuracy. Bias was defined as Cin minus eGFR and expressed in ml/min/1.73 m^2. %precision error was defined as bias/Cin x 100% and absolute %precision error was defined as |bias|/Cin x 100%, both of which are presented with standard deviation as a measure of spread of the precision error. P_{10} accuracy and P_{30} accuracy were defined as the percentage of measurements where eGFR was within ± 10% or ± 30% of Cin, respectively. Both the individual equations and combinations of different creatinine and cystatin C-based equations were analyzed.

To study the performance of the combined equations as the outcome of the creatinine and the cystatin C based equations diverged, we calculated delta-eGFR as the *absolute* difference between the creatinine-based eGFR and the cystatin-based eGFR as a percentage of the mean of the two.

(v) |%Delta-eGFR-FASage|
=|(FASage – Schwartz$_{cys}$)| / (0.5 x (FASage + Schwartz$_{cys}$))

(vi) |%Delta-eGFR-Schwartz$_{crea}$|
=|(Schwartz$_{crea}$ – Schwartz$_{cys}$)| / (0.5 x (Schwartz$_{crea}$ + Schwartz$_{cys}$))

Differences in accuracy were compared using non-parametric tests, a p-value ≤ 0.05 was considered statistically significant.

Results

Study population

Between 2004 and 2015, 408 inulin clearance tests were done in children ranging from two to 19.5 years in age. Mean age was 12.5 years with a standard deviation (SD) of 4.9, 60% were male. Cin ranged from 13.4 to 185.0 ml/min/1.73m^2, with a mean of 91.2 ml/min/1.73m^2 (SD 30.3). Underlying diagnoses were active malignancy in 23.5%, single functioning kidney in 24.0%, nephritis in 17.6%, urological disease in 10.3%, neural tube

defects in 9.3%, follow-up after malignancy in 3.4% and others in 11.8%.

Performance of equations

The bias, %prediction error, absolute %prediction error and P_{10} and P_{30} accuracy for each of the three individual equations, for the creatinine- and cystatine C-based equation pairs and for CKiD3 are summarized in table 1. For both equation pairs, accuracy was higher than that of the individual equations. P_{30} accuracy of the most accurate single equation (i.e. $Schwartz_{crea}$) was significantly lower than the combination (FASage+$Schwartz_{Cys}$)/2 (81.9 vs. 89.2%, p = 0.000 McNemar test). P_{30} accuracy of (FASage+$Schwartz_{cys}$)/2 was not significantly different from ($Schwartz_{crea}$+$Schwartz_{Cys}$)/2 (p = 1.000), nor from that of CKiD3 (p=0.189). P_{10} accuracy of (FASage+$Schwartz_{cys}$)/2 trended to be higher than that of ($Schwartz_{crea}$+$Schwartz_{Cys}$)/2 (p=0.06) and was not significantly different from CKiD3.

Table 1: Performance of all four equations and the mean of creatinine- and cystatin C-based equations; bias in ml/min/1.73m^2; % prediction error and absolute % prediction as percentage of Cin; P_{30} accuracy as percentage of cases within ±30% of Cin; P_{10} accuracy as percentage of cases within ±10% of Cin

Equation	Bias	% prediction error [SD]	Absolute % prediction error	P_{30} accuracy	P_{10} accuracy
$Schwartz_{cys}$	13.6	11.2 [20.7]	23.6 [14.0]	81.4	30.9
$Schwartz_{crea}$	5.6	3.5 [29.7]	19.3 [22.7]	81.9	36.0
FASage	-11.5	-13.2 [42.7]	23.6 [38.0]	79.7	35.5
($Schwartz_{cys}$ + $Schwartz_{crea}$)/2	9.6	7.4 [20.6]	16.6 [14.3]	89.0	35.5
($Schwartz_{cys}$ + FASage)/2	1.1	-1.0 [25.6]	16.1 [20.0]	89.2	43.6
CKiD3	2.4	0.2 [19.7]	14.2 [13.6]	90.9	47.5

The two combinations of creatinine and cystatin C-based equations, along with CKiD3 were compared across different age groups (Table 2). All three performed comparably in the different age groups. The same held true for comparison between different GFR groups based on CKD stages, i.e. stage 1; ≥90 ml/min/1.73 m^2, stage 2; 60-89 ml/min/1.73 m^2, stage 3-5; ≤ 59 ml/min/1.73 m^2 (Table 3)

Table 2: Performance of all combined equations stratified by age category. Age is given in years; n is the number of patients per age category; % prediction error is in % of Cin; P_{30} and P_{10} are percentage of cases within ±30% and ±10% of Cin, respectively

Age	n	(Schwartz$_{cys}$ + Schwartz$_{crea}$)/2			(Schwartz$_{cys}$ + FASage)/2			CKiD3		
		% prediction error [SD]	P_{30}	P_{10}	*% prediction error [SD]*	P_{30}	P_{10}	*% prediction error [SD*	P_{30}	P_{10}
2-4	36	-1.7 [32.6]	86.1	30.6	-4.0 [44.2]	86.1	25.0	-1.1 [23.6]	94.4	41.7
4-6	19	7.0 [15.0]	94.7	57.9	6.3 [15.1]	94.7	52.6	3.1 [13.8]	94.7	52.6
6-8	36	3.3 [26.2]	83.3	44.4	-0.9 [28.8]	83.3	44.4	-0.9 [24.8]	86.1	47.2
8-10	30	7.0 [17.8]	90.0	40.0	0.6 [20.9]	86.7	50.0	2.8 [17.3]	90.0	60.0
10-12	56	5.3 [17.1]	92.9	48.2	-0.5 [18.6]	91.1	51.8	-1.7 [17.5]	92.9	53.6
12-14	38	8.3 [17.4]	94.7	26.3	3.3 [15.6]	97.4	47.4	0.0 [17.0]	92.1	47.4
14-16	58	12.0 [15.2]	89.7	31.0	4.5 [16.3]	93.1	43.1	2.8 [17.3]	94.8	36.2
16-18	101	8.3 [21.3]	83.2	25.7	-6.3 [30.2]	85.1	40.6	-1.6 [22.4]	87.1	48.5
>18	34	13.3 [14.0]	97.1	41.2	-2.3 [19.0]	91.2	44.1	3.4 [15.4]	91.2	47.1

Table 3: Performance of all combined equations stratified by category of CKD. GFR is given in ml/min/1.73 m^2; n is the number of patients per age category; % prediction error is in % of Cin; P_{30} and P_{10} are percentage of cases within ±30% and ±10% of Cin, respectively

GFR	n	(Schwartz$_{cys}$ + Schwartz$_{crea}$)/2			(Schwartz$_{cys}$ + FASage)/2			CKiD3		
		% prediction error [SD]	P_{30}	P_{10}	% prediction error [SD]	P_{30}	P_{10}	*% prediction error [SD*	P_{30}	P_{10}
≥90	227	14.2 [18.6]	86.3	22.0	3.9 [25.5]	89.0	38.3	5.8 [17.5]	93.0	41.4
60-89	124	2.4 [17.9]	96.0	56.5	-5.9 [25.7]	92.7	54.0	-5.6 [19.7]	91.1	58.1
≤59	57	-9.2 [21.8]	84.2	43.9	-9.5 [21.8]	82.5	42.1	-9.3 [20.6]	82.5	49.1

Table 4 summarizes the performance of the combined equations and CKiD3 per diagnosis category. Here, the (FASage+Schwartz$_{cys}$)/2 performed poorly in the group with neural tube defects compared to (Schwartz$_{crea}$+Schwartz$_{Cys}$)/2 and CKiD3. For other diagnoses distributions are similar.

Delta-eGFR

Bias of the individual equations and the mean of the two were plotted against |%delta-eGFR| for FASage and Schwartz$_{crea}$ (Figures 1a and 1b). With increasing |%delta-eGFR|, an increase in the bias of the cystatin C-based equation was observed, indicating progressive underestimation of GFR by cystatin C as well as a decrease in the bias of the creatinine-based equation, indicating progressive overestimation of GFR by serum creatinine. This

Table 4: Performance of all combined equations stratified by diagnosis. N is the number of patients per category; % prediction error is in % of Cin; P_{30} and P_{10} are percentage of cases within ±30% and ±10% of Cin, respectively

Diagnosis	n	(Schwartz$_{cys}$ + Schwartz$_{crea}$)/2			(Schwartz$_{cys}$ + FASage)/2			CKiD3		
		% prediction error [SD]	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*
Malignancy	96	7.9 [20.0]	87.5	34.4	1.8 [23.8]	86.5	41.7	1.5 [19.4]	90.6	42.7
Single kidney	98	8.0 [16.5]	91.8	38.8	0.6 [16.4]	94.9	44.9	-0.3 [16.9]	92.9	54.1
Nephritis	72	10.4 [27.5]	83.3	13.9	1.6 [33.5]	90.3	38.9	3.4 [23.5]	88.9	33.3
Urological	42	8.4 [15.8	92.9	47.6	5.8 [12.9]	97.6	50.0	4.3 [13.0]	95.2	59.5
Neural tube defect	38	-3.5 [25.6]	84.2	39.5	-25.5 [38.8]	68.4	31.6	-13.4 [26.5]	76.3	47.4
Follow-up after malignancy	14	4.0 [13.9]	100	64.3	-3.2 [13.9]	92.9	57.1	-1.4 [12.6]	100	64.3
Other	48	8.9 [16.2]	91.7	41.7	0.5 [18.6]	89.6	52.1	1.7 [15.3]	95.8	50.0

was confirmed using linear regression with |%delta-eGFR-FASage| as dependent variable, in which B-value for the bias of FASage was -0.412 with 95% confidence interval of -0.444 to -0.380 and the B-value for bias of Schwartz$_{cys}$ was 0.486 with 95% confidence interval of 0.419 to 0.513. Since the bias of the creatinine-based equations may reflect alterations in muscle mass, such as in patients with neural tube defects or active malignancy, this analysis was repeated after excluding patients with neural tube defects or active malignancy. Still, the pattern remained unchanged (data not presented).

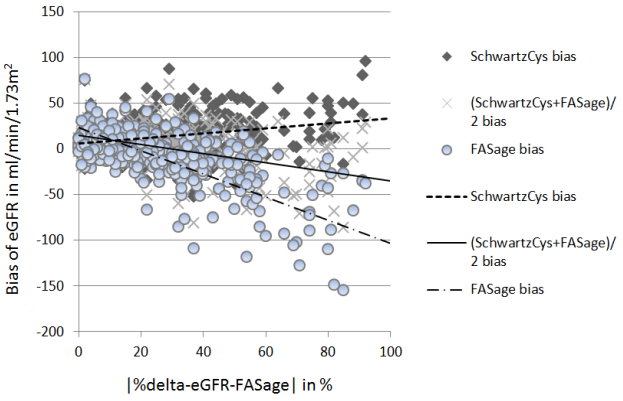

Figure 1a: Scatterplot with |%delta-eGFR-FASage| on the x-axis and bias with respect to Cin in ml/min/1.73m^2 on the y-axis
Data presented for FASage, Schwartz$_{cys}$ and the mean of the two with the corresponding linear regression lines.

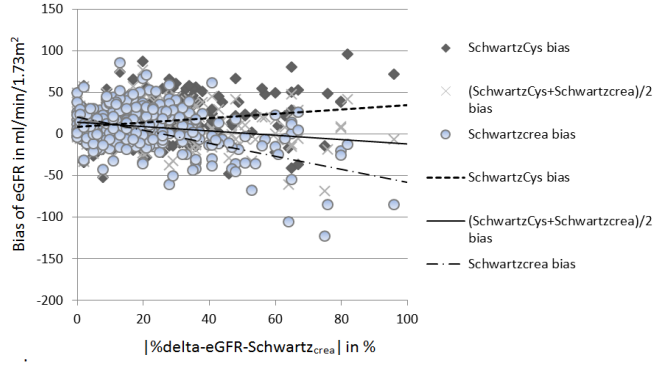

Figure 1b: Scatterplot with |%delta-eGFR- Schwartz$_{crea}$ | on the x-axis and bias with respect to Cin in ml/min/1.73m^2 on the y-axis
Data presented for Schwartz$_{crea}$, Schwartz$_{cys}$ and the mean of the two with the corresponding linear regression lines.

P_{30} and p_{10} accuracy of the same equations were compared after stratification for |%delta-eGFR| (Tables 5a and b). There is a sharp decline in accuracy of the individual equations when the difference between both estimates exceeds 30%, whereas both the mean of the two and CKiD3 have acceptable accuracy as long as |%delta-eGFR| is below 40% (p_{30} 92.5% for (FASage+Schwartz$_{cys}$)/2, 89.9% for (Schwartz$_{crea}$+Schwartz$_{cys}$)/2, 92.2 for CKiD3 respectively). Comparing |%delta-eGFR-FASage| and |%delta-eGFR-Schwartz$_{crea}$| there is less deviation between Schwartz$_{crea}$ and Schwartz$_{cys}$ compared to FASage and Schwartz$_{cys}$. At the same time, accuracy of Schwartz$_{crea}$ and Schwartz$_{cys}$ as well as the average of the two tends to be lower in the respective |%delta-eGFR| categories compared to FASage and Schwartz$_{cys}$.

Table 5a: P_{30} and P_{10} accuracy rates for FASage, Schwarz$_{Cys}$, (Schwart$_{Cys}$+FASage)/2 and CKiD3, for different categories of |%delta-eGFR-FASage|

|%Delta-eGFR|	n	FASage		Schwartz$_{cys}$		(Schwartz$_{cys}$+FASage)/2		CKiD3	
		P_{30}	P_{10}	P_{30}	P_{10}	P_{30}	P_{10}	P_{30}	P_{10}
< 10	99	93.9	44.4	96.0	46.5	96.0	47.5	94.9	50.5
10-19	92	93.5	43.5	93.5	42.4	93.5	47.8	91.3	56.5
20-29	67	95.5	49.3	79.1	19.4	92.5	46.3	94.0	53.7
30-39	61	70.5	19.7	77.0	23.0	85.2	44.3	86.9	41.0
≥40	89	43.8	18.0	57.3	15.7	77.5	32.6	86.5	34.8

Table 5b: P_{30} and P_{10} accuracy rates for Schwartz$_{crea}$, Schwartz$_{Cys}$ and (Schwartz$_{Cys}$+ Schwartz$_{crea}$)/2, for different categories of |%delta-eGFR-Schwartz$_{crea}$|

|%Delta-eGFR|	n	Schwartz$_{crea}$		Schwartz$_{cys}$		(Schwartz$_{cys}$ + Schwartz$_{crea}$)/2		CKiD3	
		P_{30}	P_{10}	P_{30}	P_{10}	P_{30}	P_{10}	P_{30}	P_{10}
< 10	156	93.6	41.0	91.0	39.7	93.6	40.4	92.9	52.6
10-19	119	82.4	38.7	89.1	33.6	87.4	37.0	93.3	52.9
20-29	59	72.9	32.2	76.3	22.0	83.1	32.2	81.4	37.3
30-39	31	77.4	29.0	64.5	25.8	93.5	25.8	90.3	35.5
≥40	43	53.5	20.9	44.2	7.0	81.4	25.6	90.7	37.2

The distribution of diagnoses across different categories of |%delta-eGFR-FASage| and |%delta-eGFR-Schwartz$_{crea}$| is shown in Figures 2a and 2b. Here, an increase in the percentage of neural tube defects is apparent with increasing |%delta-eGFR-FASage|, while active malignancy was associated with a higher |%delta-eGFR-Schwartz$_{crea}$|.

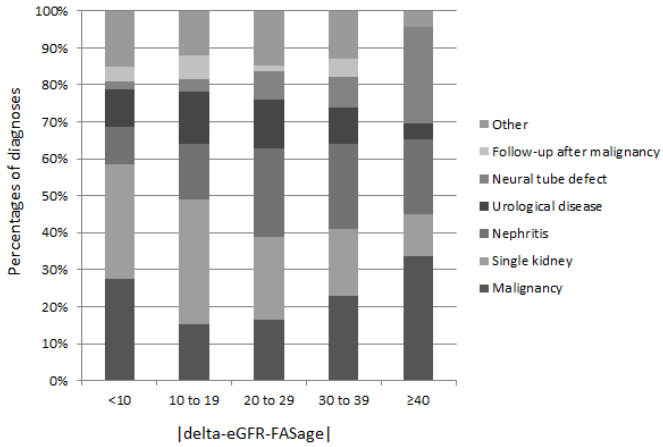

Figure 2a: Distribution of diagnoses across different levels of |%delta-eGFR-FASage|

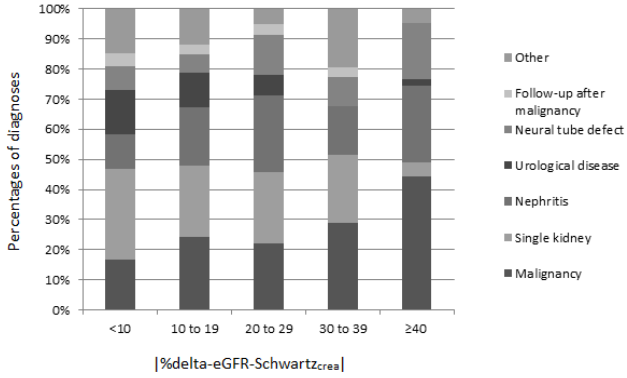

Figure 2b: Distribution of diagnoses across different levels of |%delta-eGFR-Schwartz$_{crea}$|

Discussion

Our data demonstrate that the average of a cystatin C and a creatinine-based equation outperforms the individual cystatin C or creatinine-based equations - no matter whether a height-*dependent* or a height-*independent* creatinine-based equation is used. In fact, the average of the height-independent approach using cystatin C and FASage performed comparably to the most complex CKiD3 equation, which also incorporates urea, gender and height.

This is in line with previous studies both in adult (15, 20) and in pediatric populations (3, 13-15), which have shown that the combination of creatinine and cystatin C for GFR estimation increases accuracy and precision compared to single marker equations. To this end, creatinine and cystatin C can be incorporated into one single equation (3, 21, 22) or by calculating the arithmetic mean between the creatinine and the cystatin C-based eGFR (13, 23). Of note, the exponent of creatinine and cystatin C in the complex equations by Schwartz et al (3) is very similar, which explains why the complex equations and the arithmetic means yield comparable results.

As first recognized by Grubb (24) using the mean of a creatinine and a cystatin C based eGFR offers the advantage of recognizing discrepant creatinine and cystatin C results, which are obscured if both parameters are used in a single equation. This may be a clue to conditions when one of the two markers fails, such as abnormal muscle mass (25) or urinoma (26) for creatinine and untreated thyroid dysfunction (27) or high-dose glucocorticoid treatment (28) for cystatin C.

As illustrated by our data, the difference between the creatinine and the cystatin C-based eGFR provides important information on the accuracy of the estimate. The lower the difference between both eGFR estimates, the higher the accuracy with a maximum P_{30} of up to 96% and P_{10} of up to 46%. With increasing |delta-eGFR|, accuracy decreases but can be maintained above 85% by using the average of the creatinine- and the cystatin C-based equation rather than the single equations. This was also observed by Björk et al in adult patients when comparing creatinine and cystatin C-based equations (29).

Both in Björk's and the present study, increasing |delta-eGFR| is associated with an underestimation of GFR by cystatin C and an overestimation of GFR by creatinine. This can in part be explained by patients with neural tube defects and active malignancy in our study accounting for overestimation of $eGFR_{crea}$. However, the association persists after these two patient groups have been excluded. A potential explanation for this finding is the "shrunken pore syndrome" proposed by Grubb et al (17), where elimination of the large cystatin C molecule (MW 13.3 kDa) is diminished compared to creatinine (MW 118 Da) due to alterations in the glomerular filtration characteristics.
In line with our earlier study (9), the FASage equation performed comparably with $Schwartz_{crea.}$ The poor performance of the FASage equation compared to $Schwartz_{crea}$ in patients with neural tube defects relates to the different correction for muscle mass in both methods. While FASage uses an age-related creatinine reference (8), $Schwartz_{crea}$ uses height as a surrogate marker for muscle mass. As children with neural tube defects have short stature (30), the use of height rather than age may to some extent have corrected for the altered body composition in spina bifida patients.

When comparing the distribution of |%delta-eGFR| based on FASage vs. $Schwartz_{crea}$, we observed less deviation between the two Schwartz-estimates. In two-thirds of the latter, the deviation was below 20% compared to 48% when using FASage and $Schwartz_{cys}$. This observation probably reflects the fact that the two Schwartz eGFRs - unlike FASage and $Schwartz_{cys}$ - were developed in the same population.(3) In fact, the distribution of |%delta-eGFR| using FASage was almost identical to Björk's findings in adults (0-10: 29%, 10-20: 24%, 20-30: 16%, 30-40: 12% and >40: 18%).(29)

This study has several limitations: (i) The population studied was that of a tertiary referral hospital, with relatively severe morbidity. Still, mean GFR was 91.2 ml/min/1.73m^2, which is at the breakpoint between CKD stage 1 and stage 2, which will be the most prevalent in a more general pediatric population. Also, as FASage is based on creatinine concentrations from healthy children, its performance can be expected to be at least as good in a primary care situation. (ii) Potential co-morbidity and the use of medication were not recorded in our dataset. For creatinine, this concerns bilirubin when using the Jaffe method (31) and drugs interfering with creatinine excretion (32), while thyroid dysfunction and glucocorticoid steroid use show relevant interactions with cystatin C. (26) With respect to oncology patients, Vermassen et al. have demonstrated falsely decreased cystatin C levels due to cathepsin D-mediated proteolysis of cystatin C induced by antiangiogenic drugs. [33] This illustrates potential interference with either of the markers, both from underlying morbidity and medication, which underscores the advantage of comparing and combining both GFR markers. (iii) During the long period of data acquisition, both creatinine and cystatin C assays were re-calibrated following introduction of new IFCC reference material. This was corrected for by using conversion factors established by the manufacturer for cystatin C and in house for creatinine. This may potentially have influenced our analysis, yet separate analysis comparing data before and after re-calibration showed no systematic difference (data not presented). (iv) Based on the performance in earlier studies of our group, we have chosen to study FASage, $Schwartz_{crea}$ and $Schwartz_{cys}$ out of a number of different eGFR equations. For example, we could also have studied Pottel's FAScys equation instead of $Schwartz_{cys}$. As the mean of FASage and FAS_{cys} performed comparably to the mean of $Schwartz_{crea}$ and $Schwartz_{cys}$ in a recent study(14), we believe that the findings presented here can be extrapolated to the combination of FAScys and FASage.

Based on our results we propose that laboratories calculate eGFR using a height-independent creatinine and a cystatin C equation and report the average of two results if |%delta-eGFR| is below 40%. If the difference between both eGFRs exceeds 40%, both eGFRs should be reported. This will alert the treating physician to search for an underlying pathology. If no explanation can be found and the discrepancy persists, a golden standard GFR measurement should be considered.

Conclusion

The combination of a cystatin C-based equation with a *height-independent* creatinine-based eGFR equation performs at least as well as the combination with the commonly used *height-dependent* equation. This facilitates direct eGFR reporting by the laboratory.

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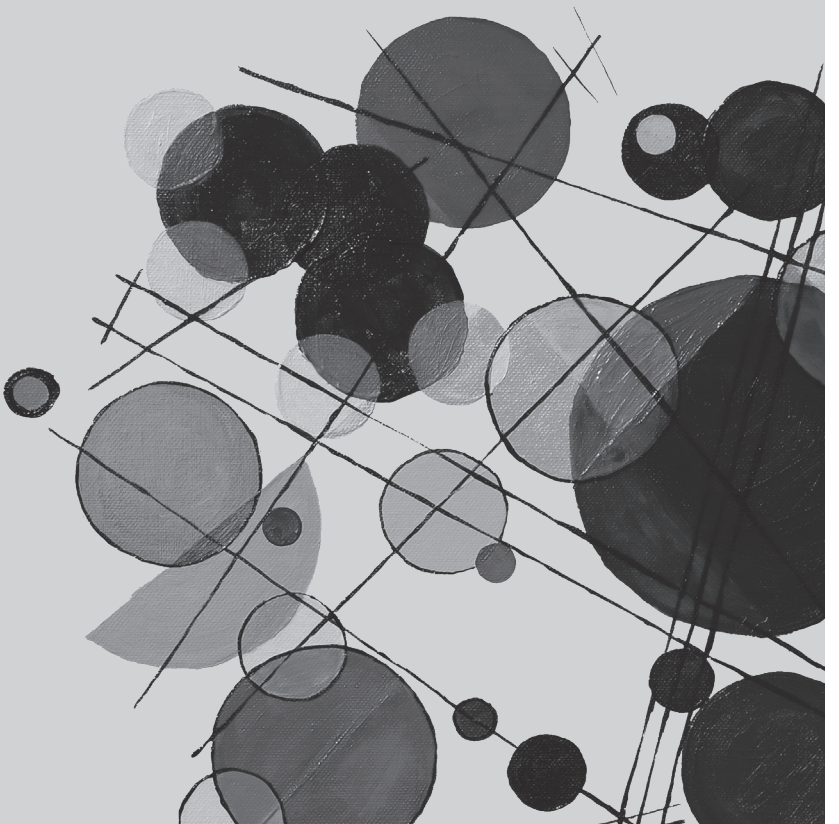

Combining GFR estimates from cystatin C and creatinine – what is the optimal mix?

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Abstract

Background

Combining estimated glomerular filtration rate (eGFR) equations based on creatinine and cystatin C has been shown to improve the accuracy of GFR estimation. This study aims to optimize this strategy for height-independent GFR estimation in children.

Methods

Retrospective study of 408 inulin clearance tests with simultaneous IFCC calibrated measurements of creatinine, cystatin C and urea in children (mean age 12.5 years, GFR 91.2 ml/min/1.73m^2) comparing the arithmetic (mean$_{arith}$) and geometric means (mean$_{geom}$) of a height independent creatinine- (FASage) and a cystatin C-based equation (FAScys), with the complex height-dependent CKiD3 equation incorporating gender, height, cystatin C, creatinine and urea.

Results

Mean$_{geom}$ had a P_{30} accuracy of 89.2% compared to mean$_{arith}$ 87.7% ($p = 0.030$) as well as lower bias and %precision error and performed almost as well as CKiD3 (P_{30} accuracy 90.9%). Modifying the weight of FASage and FAScys when calculating the means showed that an equal contribution was most accurate in most patients. In spina bifida patients FAScys alone outperformed any combination. Malignancy or nephritis patients had slightly higher accuracy with weighted means favoring cystatin C or creatinine, respectively. Disagreement between FAScys and FASage was inversely correlated with the accuracy of mean$_{geom}$. When disagreement exceeds 40%, application of weighted means based on diagnosis improves performance of eGFR.

Conclusions

In the absence of height data the optimal strategy for estimating GFR in children is by using the geometric mean of FASage and FAScys. When there is large disagreement between the two, weighted means based on diagnosis improves accuracy.

Keywords

Height-independent, eGFR, creatinine, cystatin C

Introduction

It is widely accepted that estimated GFR (eGFR) rather than the serum concentration of endogenous marker markers of GFR should be used in the diagnosis of kidney disease as it leads to earlier recognition of impaired kidney function and better follow-up during treatment.(1) While cystatin C concentrations are independent of body composition (2, 3), creatinine concentrations are strongly affected by muscle mass. In children, height is a suitable covariate to correct for increasing muscle mass during growth and forms part of most creatinine-based eGFR equations.(4, 5) FASage (Full Age Spectrum, based on age), the only creatinine-based eGFR equation for children, which does not require anthropometric data was developed by Pottel et al.(6) He related the individual creatinine reading to the age-specific reference values of creatinine and GFR. Their group applied the same technique to cystatin C (FAScys).(7) Several other cystatin C based equations have been developed which also do not incorporate anthropometric data.(8-10).

Multiple studies both in adults and in children have demonstrated that a combined approach using both creatinine and cystatin C improves GFR estimation. This can be done either by calculating the mean between a creatinine-based and the cystatin C-based eGFR (11-14) or by establishing complex equations including both markers. (4, 15) The two best-performing complex equations i.e. CKiD3 for children (4) and i.e. CKD-Epi for adults (15) were developed using linear regression on logarithmic data. As a consequence, they both incorporate cystatin C and creatinine using an exponent rather than a coefficient.

This led us to hypothesize that using the geometric ($mean_{geom}$) rather than the arithmetic mean ($mean_{arith}$) when combining eGFR equations may further improve accuracy. We also sought to explore whether modifying the contribution of the cystatin C- and the creatinine-based eGFR when calculating the mean might further improve accuracy. The ultimate goal of this study is to propose a strategy for height-independent GFR estimation that yields optimum accuracy and is suitable for daily clinical practice.

Materials and methods

Materials

We retrospectively analyzed data from single injection inulin clearance (Cin) studies performed on clinical grounds, or as part of Institutional Review Board-approved studies between 2004 and 2017 in a single medical centre, where creatinine, cystatin C and urea had been measured simultaneously. Details on data collection and analytical procedures have

been published previously.(11) GFR was measured by the inulin single-injection method, this method has been described in detail by Westland et al; (16) all patients received a single intravenous dose (5000 mg/1.73m² of body surface area with a maximum dose of 5000 mg) of inulin (Inutest®, Fresenius, Bad Homburg, Germany), which was administered within 1 minute. Serial blood samples were obtained at 10, 30, 90 and 240 minutes after injection. Immediately after sampling, blood was centrifuged at 3000 rotations per minute for 10 minutes and stored at -20°C until measurement. Inulin was measured within 14 days using an enzymatic method based on the determination of fructose after acid hydrolysis of inulin as described by Jung et al (17) with some minor modifications.(18) GFR-inulin (ml/min/1.73m²) was calculated with MW/Pharm 3.5 software (Mediware, Groningen, The Netherlands), a pharmacokinetic program using a Bayesian estimate from patient and population data.8 In short after receiving a single dose of 5000 mg/ 1.73 m^2 inulin with a maximum of 5000 mg intravenously, serial serum measurements of inulin were done after 10, 30, 90 and 240 minutes. From the rate of decline in serum levels the GFR was calculated.(19) Serum creatinine was measured using the IDMS traceable creatinase/sarcosine oxidase enzymatic method from 2008 onwards.(20) Before 2008, a kinetic Jaffe method was used, which was subsequently converted to fit the later IDMS traceable method by a conversion equation established locally (IFCC creatinine = old creatinine x 1.1 – 26). For cystatin C a particle-enhanced immunonephelometric assay (PENIA; Siemens Healthcare, Marburg, Germany) on a Behring Nephelometer II was used in accordance with the method used by Schwartz et al.(4) Measurements performed after IFCC-calibration of the Siemens assay were divided by 1.17 to fit the original calibration of the cystatin C-based Schwartz equation. Conversely measurements performed before IFCC-calibration were multiplied by 1.17 to fit calibration for the other cystatin C-based equations.(21)
Patient characteristics were extracted from patient charts and entered into a blinded database for statistical analysis.

Equations
For creatinine, we used the most recent FASage equation, a height independent equation based on age-specific normal values.(6)

(i) FASage [ml/min/1.73m^2] = 107.3 / (creatinine/Q)
with creatinine concentration in mg/dl and Q being the age-specific normal value of creatinine

Additionally for comparison of the performance, we included the FASheight equation in the supplements.(22)

(ii) FASheight [ml/min/1.73m^2] = 107.3 / (creatinine/Q)
with creatinine concentration in mg/dl and Q being the height-specific normal value of creatinine, calculated as Q = 3.94 -13.4 x height + 17.6 x height^2 – 9.84 x height^3 + 2.04 height^4

For cystatin C, we chose equations which were developed at least in part in children using IFCC calibrated (23) cystatin C measurements (i.e. CAPA (8) and FAScys (7)) or which were traceable to IFCC standards, i.e. Schwartz$_{cys.}$.(4)

(iii) Schwartz$_{cys}$ [ml/min/1.73m^2] = 40.6 x (1.8/cystatin C)$^{0.93}$
with cystatin C concentrations in mg/l

(iv) CAPA [ml/min/1.73m^2] = 130 x cystatin C$^{-1.069}$ x age$^{-0.117}$ - 7
with cystatin C concentrations in mg/l and age in years

(v) FAScys [ml/min/1.73m^2]= 107.3/(cystatin C/0.82)
with cystatin C in mg/l and 0.82 being the normal value above two years of age

We calculated the arithmetic mean between FASage and FAScys as

(vi) mean$_{arith}$ = 0.5 (FASage + FAScys)

and the geometic mean as

(vii) mean$_{geom}$ = (FASage x FAScys)$^{0.5}$

For reference we compared the combinations of these equations with the complex CKiD3 equation, which combines gender, height, creatinine, cystatin C and urea (4) as well as the combined FAS equation using cystatin C and creatinine without correction for height (FAScombined).(7)

(vii) CKiD3 [ml/min/1.73m^2] = 39.8 (ht/creatinine)$^{0.456}$ x (1.8/cystatin C)$^{0.418}$ x (30/urea)$^{0.079}$ x 1.076^{male} x (ht/1.4)$^{0.179}$
with height in meters, creatinine concentration in mg/dl, cystatin C concentration in mg/l, urea concentration in mg/dl and male gender coded as 1 and female gender as 0

(ix) FAScombined [ml/min/1.73m^2] = 107.3/ ((0.5xcystatinC/0.82)+(0.5xcreatinine/Q)) with cystatin C in mg/l, creatinine concentration in mg/dl and Q being the age specific normal value of creatinine

Statistics

In extension of previous studies using the arithmetic means (mean$_{arith}$) of creatinine- and the cystatin C- based equations,(11, 12) we now studied the geometric mean of the creatinine- and the best performing cystatin C-based equation (mean$_{geom}$). We also assessed the effect of changing the relative contribution of cystatin C and creatinine in the population as a whole and in subpopulations.

The performance of different equations was analyzed by calculating bias, %precision error, absolute %precision error as well as P_{10} and P_{30} accuracy. Bias was defined as eGFR minus Cin and expressed in ml/min/1.73 m^2. %precision error was defined as bias/Cin x 100% and absolute %precision error was defined as |bias|/Cin x 100%. These data are presented as means [SD]. P_{10} and P_{30} accuracy describes the proportion of eGFR measurements within ±10% and ±30 % of Cin, respectively and is given in %.

Performance was further assessed in subgroups based on CKD stage, diagnosis and ΔGFR, the latter being defined as (FASage-FAScys)/(0.5 x (FASage+FAScys)).

ΔGFR forms the basis of the "Lund approach", first described by Grubb et al.(24) If the cystatin C- and the creatinine-based estimates differ by more than 40% Grubb suggests to search for patient-specific conditions which interfere with cystatin C (e.g. thyroid dysfunction, glucocorticoid therapy) of creatinine metabolism (e.g. muscle wasting) (25) and estimate GFR using the most suitable marker.

Weighting of the two equations was explored by comparing the P_{30} and P_{10} accuracy rates for different levels of α and β, i.e. (α x FASage + β x FAScys) for mean$_{arith}$ and (FASage$^{\alpha}$ x FAScys$^{\beta}$) for mean$_{geom}$ respectively, where α ranged from 0 to 1 and α+β=1.
Comparison between groups was done using paired and unpaired t-tests for continuous data and the McNemar test for the accuracy data. P-values below 0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 23.

Results

Study population

408 inulin clearance tests were analyzed in 319 unique children and adolescents between the age of two and 19.5 years. Characteristics of the study population have been described in detail previously (11). In short, mean GFR was 91.2 ml/min/1.73m^2 [30.3], mean age was 12.5 years [4.9], 60% were male. The spectrum of diagnoses included single kidney (n=98), malignancy (n=96), nephritis (n=72), urological abnormality (n=42), neural tube defect (n=38), follow-up after malignancy (n=14) and other (n=48).

Performance of equations

All four single marker equations and the arithmetic and geometric means of FASage and FAScys were compared to CKiD3 with respect to bias, % precision error, absolute %precision error and P_{10} and P_{30} accuracy rates. FAScys was chosen because it was the best performing single cystatin C-based equation. As shown in Table 1, all single equations were inferior to the means and to the complex equations. P_{30} accuracy of $mean_{geom}$ was significantly higher than $mean_{arith}$ (89.2 vs. 87.7%; p=0.031) while P_{10} accuracy tended to be higher for $mean_{arith}$ (46.3 vs 44.4%; p=0.057). Performance of CKiD3 tended to be slightly better for all parameters compared to the means and to FAScombined, but this did not reach statistical significance in terms of accuracy. When compared to $mean_{geom}$, p-values for CKiD3 were 0.143 and 0.218 for P_{30} accuracy and P_{10} accuracy, respectively, and 0.109 and 0.424 for FAScombined. Separate analysis was done in the subgroup of primary cases, which yielded very similar results, albeit slightly higher accuracy rates for all equations (data not presented).

To further characterize the performance of the means compared to CKiD3, the results for $mean_{geom}$ and $mean_{arith}$ were stratified for CKD stage (Table 2) and diagnosis (Table 3). Here, %prediction error and accuracy tended to be poorer in CKD stage 3-5 for all three methods, although this only reached statistical significance for P_{30} accuracy of CKiD3 comparing CKD stage 1 with stage 3-5 (p=0.014). When stratified for diagnoses poor performance was evident in the group with neural tube defects. After excluding neural tube defects, P_{30} accuracy of $mean_{geom}$ and $mean_{arith}$ rose to 91.6 and 90.8%, respectively, and of CKID3 to 92.4%. Accuracy was not significantly different between CKiD3, $mean_{geom}$ and $mean_{arith}$ in the subgroups. Similar results were found when repeating the analysis with the FASheight equation, which can be seen as supplemental Table 1, 2 and 3.

Table 1: Performance of the different equations
Data are presented as means [standard deviation]. Bias is expressed in ml/min/1.73m^2, %prediction error and absolute %prediction error in % of Cin. P_{30} and P_{10} accuracy rates are in %. Statistical significance compared to $mean_{geom}$. * = p < 0.05, ** = p < 0.01

Equation	Bias [SD]	%prediction error [SD]	Absolute %prediction error [SD]	P_{30} accuracy	P_{10} accuracy
FAScys	-7.1 [20.5]**	-4.4 [22.4]**	17.0 [15.3]**	83.6**	39.0
$Schwartz_{cys}$	-13.6 [20.1]**	-11.2 [20.7]**	23.6 [14.0]**	81.4**	30.9**
CAPA	-4.0 [22.3]**	-3.2 [25.6]**	19.6 [16.7]**	79.4**	31.4**
FASage	11.5 [42.4]**	13.2 [42.7]**	23.6 [38.0]**	79.7**	35.5**
$mean_{arith}$	2.2 [25.1]**	4.4 [26.3]**	16.2 [21.1]*	87.7*	46.3
$mean_{geom}$	0.5 [21.2]	2.6 [22.7]	15.4 [16.9]	89.2	44.4
CKiD3	-2.4 [18.5]**	-0.2 [19.7]**	14.2 [13.6]**	90.9	47.5
FAScombined	-1.1 [19.4]**	1.0 [20.9]**	14.9 [14.6]	90.7	43.4

Table 2: Performance of $mean_{arith,}$ $mean_{geom}$ and CKiD3 in different CKDcategories
N denotes the number of patients per category. %prediction error is in % of Cin and presented as mean [standard deviation]. P_{30} and P_{10} denote the proportion of cases within ±30% and ±10% of Cin and are expressed in %.

GFR	N	$mean_{arith}$			$mean_{geom}$			CKiD3		
		% prediction error [SD]	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*
≥90	227	-0.1 [26.1]	88.1	45.8	-2.1 [21.2]	89.9	43.6	-5.8 [17.5]	93.0	41.4
60-89	124	9.6 [26.6]	89.5	50.0	8.0 [23.4]	90.3	48.4	5.6 [19.7]	91.1	58.1
≤59	57	11.0 [23.3]	82.5	40.4	9.4 [23.1]	84.2	38.6	9.3 [20.6]	82.5	49.1

Table 3: Performance of $mean_{arith,}$ $mean_{geom}$ and CKiD3 in different diagnoses
N denotes the number of patients per category. %prediction error is in % of Cin and presented as mean [standard deviation]. P_{30} and P_{10} denote the proportion of cases within ±30% and ±10% of Cin and are expressed in %.

Diagnosis	N	$mean_{arith}$			$mean_{geom}$			CKiD3		
		% prediction error [SD]	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*	*% prediction error [SD]*	*P_{30}*	*P_{10}*
Malignancy	96	1.8 [24.3]	85.4	42.7	-0.6 [22.0]	87.5	40.6	-1.5 [19.4]	90.6	42.7
Single kidney	98	2.9 [16.9]	93.9	54.1	2.3 [16.7]	93.9	52.0	0.3 [16.9]	92.9	54.1
Nephritis	72	1.1 [33.9]	87.5	45.8	-1.7 [24.2]	88.9	40.3	-3.4 [23.5]	88.9	33.3
Urological	42	-3.4 [13.3]	100	50.0	-3.9 [13.3]	100	50.0	-4.3 [13.0]	95.2	59.5
Neural tube defect	38	30.1 [39.9]	57.9	28.9	25.5 [34.2]	65.8	31.6	13.4 [26.5]	76.3	47.4
Follow-up after malignancy	14	7.0 [14.3]	92.9	50.0	-6.6 [14.2]	92.9	50.0	1.4 [12.6]	100	64.3
Other	48	2.9 [19.6]	91.7	47.9	2.2 [19.5]	91.7	45.8	-1.7 [15.3]	95.8	50.0

ΔeGFR has been shown to be a major predictor of bias and accuracy, with accuracy decreasing and bias increasing as ΔeGFR increases.(11) This is illustrated in Figure 1 by plotting P30 accuracy of $mean_{geom}$ against ΔeGFR as a continuous variable. Here, a steep drop in accuracy is seen above a ΔeGFR of 40% indicating this as a critical value when considering the validity of a GFR estimate. This applied to only 71 out of the 408 studies.

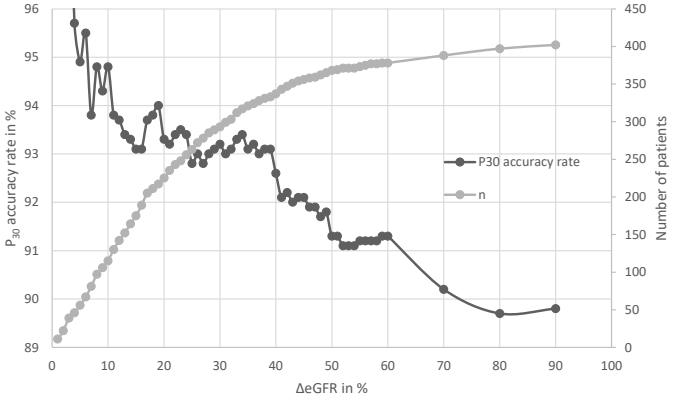

Figure 1: P30 accuracy at different levels of ΔeGFR
P_{30} accuracy rates of $mean_{geom}$ plotted against ΔeGFR for the entire group. N denotes the cumulative number of patients.

Weighting of the equations

The P_{30} and P_{10} accuracy rates are plotted against levels of α in Figures 2 to 4. In this analysis α = 1 represents eGFR based solely on creatinine, i.e. FASage, whereas α = 0 represents eGFR based solely on cystatin C, i.e. FAScys. There is a clear parabolic relationship of P_{30} and P_{10} accuracy rates for both the geometric and the arithmetic means in the total group with a modest peak at an α-value of about 0.4. Patients with neural tube defects deviate from this pattern as accuracy increases linearly with diminishing contribution of creatinine (Figure 2). In these patients FAScys clearly out-performs all equations incorporating creatinine. Restricting our analysis to the group without neural tube defects we found a parabolic relationship with a broad peak between α-values of 0.3 and 0.7. Of note, in almost all analyses, the peak in P_{30} accuracy is at a slightly lower α-value than in P_{10} accuracy. In the malignancy group P_{30} accuracy was maximal at an α of 0.4, while this was at 0.7 in the nephritis patients (Figure 3). We also assessed the impact of glucocorticosteroid use (Figure 3) and different levels of ΔeGFR (Figure 4).

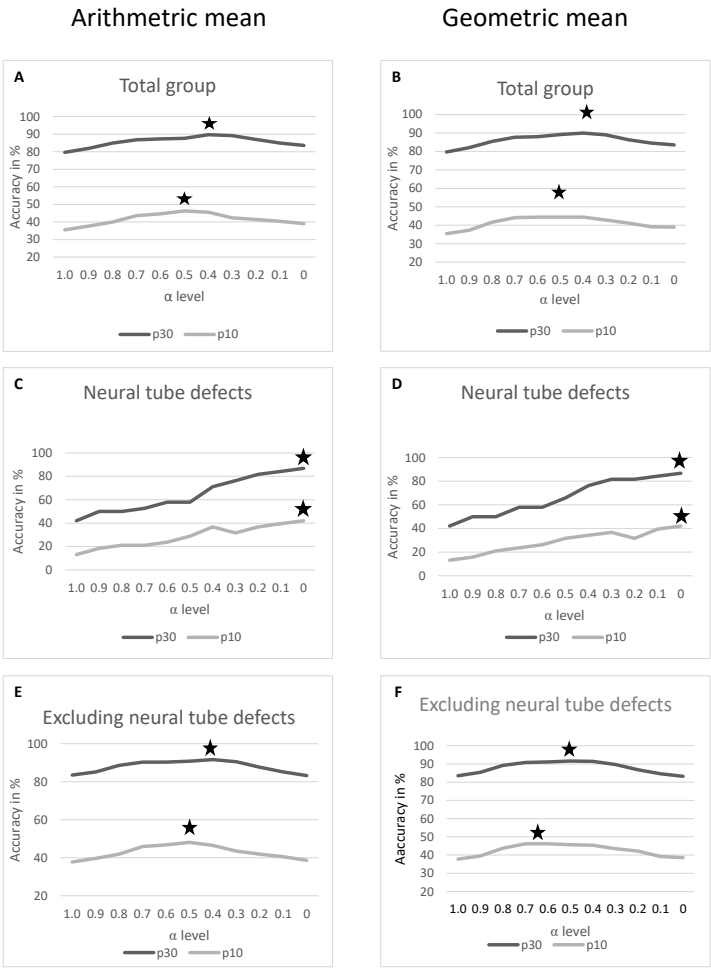

Figure 2: Accuracy of means with varying contribution of creatinine and cystatin C
Comparison between the $mean_{arith}$ (left column) and $mean_{geom}$(right column). Analysis for entire group, patients with neural tube defects and patients without neural tube defects. P_{30} and P_{10} accuracy with changing α, where α = 1 is equivalent to FASage and α = 0 to FAScys. The asterisk indicates maximum accuracy.

At low levels of ΔeGFR, changing α had little impact on accuracy, i.e. FASage and FAScys were both highly accurate and combining them added little benefit. At higher levels of ΔeGFR, however, combining the markers improved accuracy. In the group with ΔeGFR 30-40, accuracy was highest around an α-value of 0.5, in the group with ΔeGFR>40 this was at an α around 0.3 to 0.4.

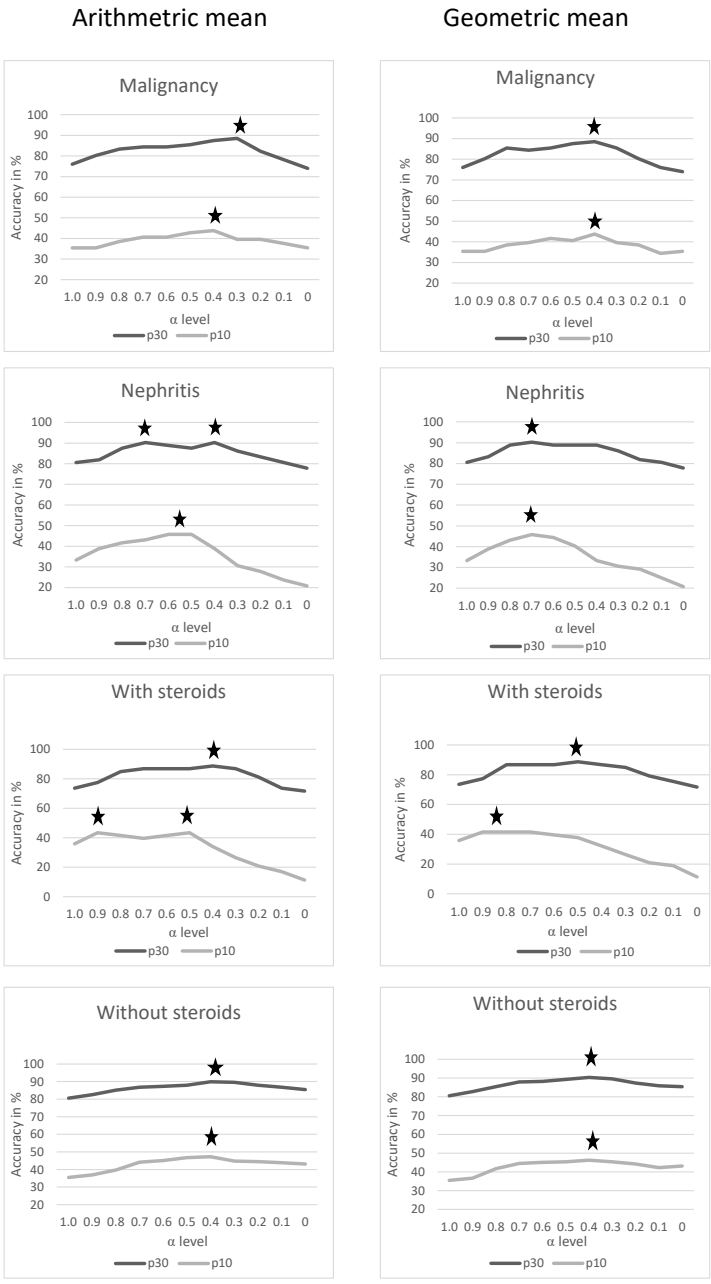

Figure 3: Accuracy of means with varying contribution of creatinine and cystatin C
Comparison between the $mean_{arith}$ (left column) and $mean_{geom}$ (right column). Analysis for patients with malignancy and patients with nephritis and patients with and without steroid treatment. P_{30} and P_{10} accuracy with changing α, where α = 1 is equivalent to FASage and α = 0 to FAScys. The asterisk indicates maximum accuracy.

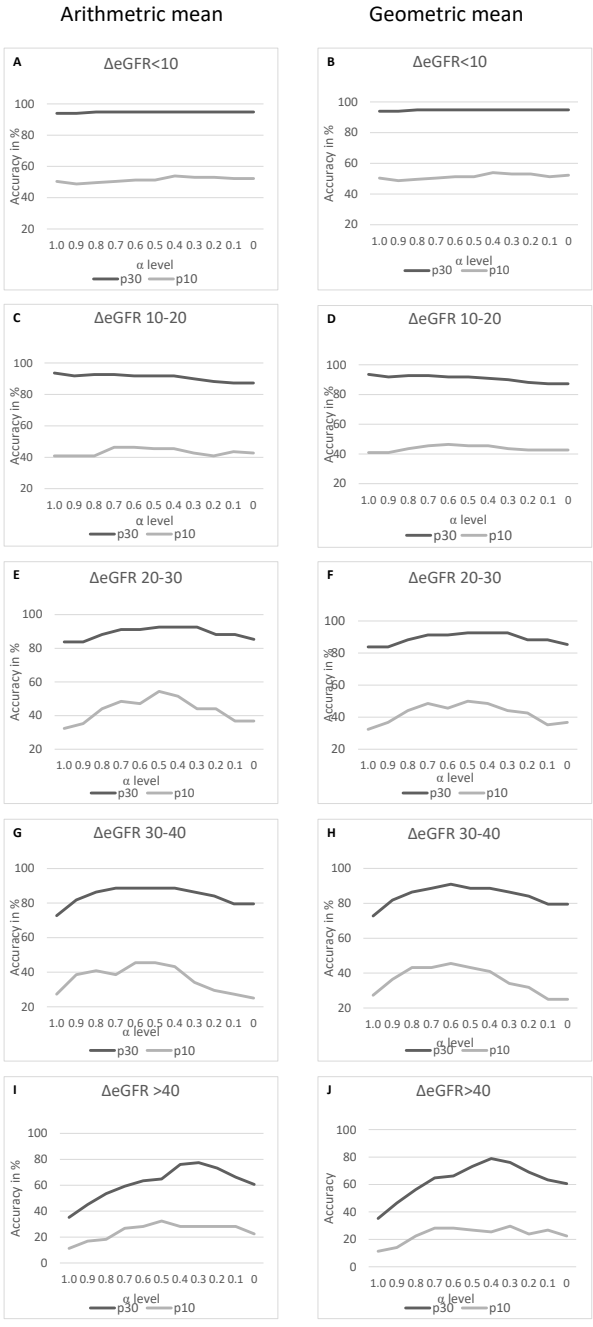

Figure 4: Accuracy of the means depending on the contribution of creatinine and cystatin C
Comparison between the $mean_{arith}$ (left column) and $mean_{geom}$(right column). Analysis of the entire patient population divided in categories of ΔeGFR. P_{30} and P_{10} accuracy with changing α, where α = 1 is equivalent to FASage and α=0 to FAScys.

The "Lund approach"

In an attempt to improve performance in the group of 71 studies with ΔeGFR>40 we checked the underlying diagnoses and opted for a weighted geometric mean as described above for studies in patients with malignancy (N = 22; $FASage^{0.4}$ x $FAScys^{0.6}$), a neural tube defect (N = 17; FAScys) and nephritis (N = 16; $FASage^{0.7}$ x $FAScys^{0.3}$). In the remaining studies (11 with single kidney, three with urological disease and two others) we used the unweighted geometric mean. Using this approach, the P_{30} accuracy of $mean_{geom}$ improved from 73.2% to 83.1% (p=0.092), P_{10} accuracy from 26.8% to 39.4% (p=0.078) and %prediction error decreased from -14.0 to -7.7 (p=0.05).

Discussion

In the present study, the mean of a cystatin C-based and a creatinine-based eGFR improved bias, precision and accuracy compared to single parameter equations. In contrast to data in adults, (13) but in line with previous reports in children (26) the geometric mean improved overall performance and specifically P_{30} accuracy when compared to the arithmetic mean in our pediatric population. Still, the differences are small and from a clinical standpoint both approaches are acceptable in the clinical setting. Of note, the geometric mean of the height-independent FASage and FAScreat equations performed almost as well as the complex CKiD3 equation, which requires anthropometric data, gender and urea concentrations. This makes our approach very attractive for direct GFR reporting by the clinical chemistry laboratory using only endogenous markers.

We studied accuracy as a function of a weighted mean favoring either eGFR based on cystatin C or creatinine. Looking at the entire group, a parabolic relationship with a broad peak between α-values of 0.3 and 0.7 was found indicating that an equal contribution of both markers is suitable in most clinical situations. This applies to the arithmetic as well as to the geometric means.

However, subgroup analysis revealed important differences, which can be used to optimize GFR estimation without the need for tailored equations for specific subpopulations.(27, 28) This is in line with a recent review by Filler et al, identifying subpopulations at risk for poor performance of the markers.(29) This is most striking in the group of patients with neural tube defects where accuracy improves steadily when increasing the weight of FAScys in the calculation. This can be explained by large differences in muscle mass in patients with neural tube defects, which greatly influence creatinine (30) but not cystatin C production.(31)

In patients treated for malignancies, the peak lies around an α-value of about 0.4 indicating that cystatin C should be weighted stronger than creatinine in this group. This is not unexpected since active malignancies tend to alter muscle mass.(32) Several authors have demonstrated that cystatin C is superior to creatinine in this population.(33, 34) Despite this, our data illustrate that it is preferable to combine both markers rather than rely on cystatin C alone as P_{30} accuracy drops below 75% at either end of the parabola (Figure 3). In nephritis patients, accuracy is lowest when only FAScys is used. Here, an α-value of about 0.6 to 0.7 favoring creatinine-based FASage yields the best results, possibly reflecting glucocorticosteroid treatment, which alters cystatin C production.(35-37) This can be seen in Figure 3, where P_{10} accuracy in particular diminishes in steroid-treated patients when α drops below 0.3 to 0.4 while it peaks around these α-values in the steroid-free patients.

In line with previous data both in children (11) and in adults (38) ΔeGFR, i.e. the difference between cystatin C- and creatinine-based eGFR, is an important predictor of accuracy of the GFR estimate. ΔeGFR exceeding 40% - which was found in 17% of our measurements - is indicative of poor accuracy. Modifying the weight of FASage and FAScys has little effect on accuracy if ΔeGFR is below 20% as both estimates are very similar to each other. This is the case in some 55% of our measurements, where P_{30} accuracy exceeds 90% and P_{10} accuracy 45%, which is close to the maximum that can be achieved when comparing estimated GFR with measured GFR.(39) When ΔeGFR is between 20 and 40%, the highest accuracy is found at an α-value around 0.5, which means that in these patients using the un-weighted mean is best, too.

The situation changes when ΔeGFR exceeds 40%. Here, the separate eGFRs should be reported and the treating clinician should weight FASage and FAScys based on diagnosis. This was first proposed by Anders Grubb, who recommended choosing either a creatinine- or a cystatin C-based equation in specific patient groups.(40) Here, our data suggest that a more sophisticated approach weighting the contribution of creatinine and cystatin C may yield even better performance. In patients with spina bifida, this should be FAScys, in patients with malignancy, $FASage^{0.4}$ x $FAScys^{0.6}$ and in nephritis patients, $FASage^{0.7}$ x $FAScys^{0.3}$. Using these coefficients in our high-risk subpopulation we observed a strong improvement in accuracy and bias, albeit not significant due to limited power. These findings call for external validation in a larger cohort.

Our study has several limitations. (i) Measurements were performed over a long period of time and both the calibration of the cystatin C assay and the method for creatinine measurement changed during that time period. Yet, the performance of the eGFR

equations is very good compared to other studies indicating that the calibration was correct. (ii) The study was performed at a tertiary nephro-urological referral center with significant morbidity and mild to moderate CKD. This means that it is not certain whether the results can be extrapolated to primary care centers. However, we expect that our findings will also be applicable in these circumstances as the performance of the means was equally good in patients with CKD stages 1 and 2. Adding to this limitation is the fact that we included some repeated clearance studies in our analysis, which may have introduced some bias by skewing the results. (iii) The number of patients with CKD 3 and higher is limited and we did not study any transplanted patients, a group which might profit from a weighted approach for GFR estimation.(27, 41, 42) Other high-risk groups, which deserve specific attention are – among others - patients with liver cirrhosis (43) or rheumatic disease.(44) (iv) Our patients are relatively old for a pediatric population. Younger children might have slightly different optimal weighting due to differences in body composition. It also remains to be demonstrated if our method holds in adults. Finally, the promising results of the "Lund approach" are likely biased due to the fact that we applied the weighting coefficients derived from the same population. Therefore, our findings need to be externally validated in a larger population. This may also allow for further refinement of the weighting proposed from our data.

Conclusion

Laboratories should report eGFR in children by calculating the geometric mean between the height-independent creatinine-based FASage and the cystatin C-based FAScys equations when ΔeGFR is smaller than 40%. If ΔeGFR is larger, the two separate eGFRs should be reported so that the treating physician can calculate a weighted mean between FASage and FAScys depending on the underlying diagnosis. This simple method can be used in daily practice on the ward and may replace complex equations calibrated for specific patient populations. If the discrepancy cannot be attributed to individual patient characteristics, the measurement should be repeated and/or a gold standard GFR measurement using an exogenous marker be considered. While this strategy yielded promising results in our hands, external validation is still required.

Conflict of interest: The authors declare that there are no conflicts of interest.

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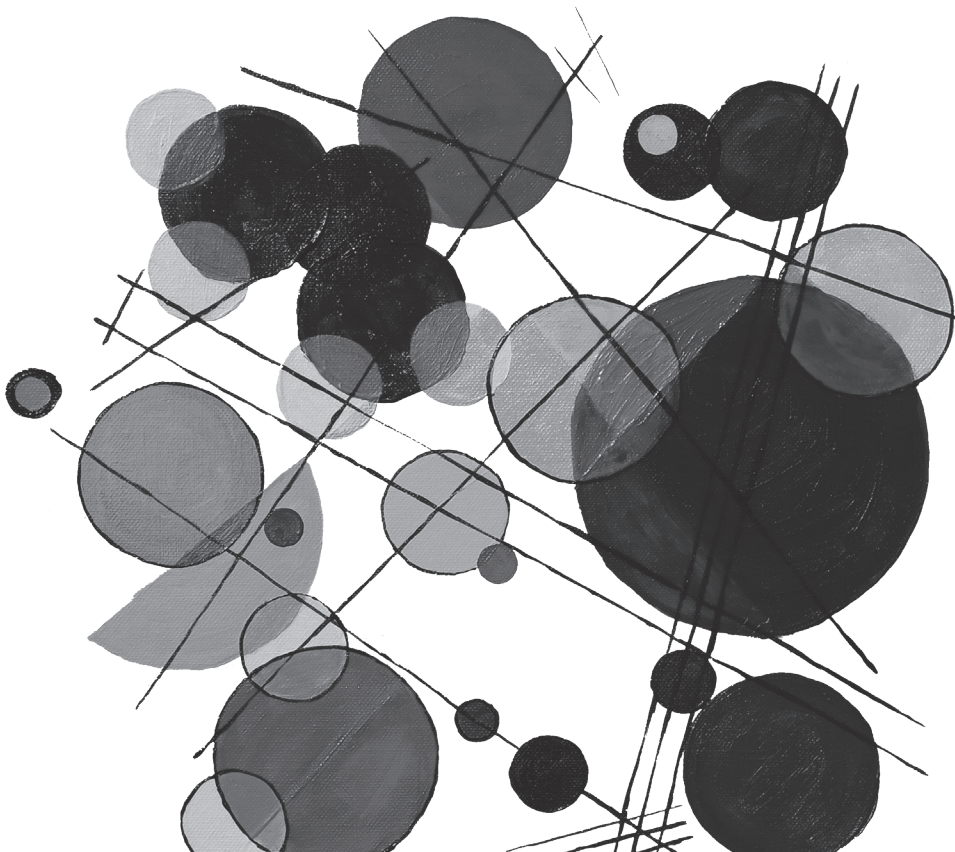
Section three

General discussion and conclusions



General discussion

The aim of this thesis was to develop a clinically applicable strategy for accurate GFR estimation in children using readily available endogenous serum markers.

The importance of accurate GFR estimation is emphasized in guidelines and clinical studies (1, 2) as it is the central parameter for the assessment of kidney function. As such it is indispensable for diagnosis and follow-up of kidney disease (3) as well as for dosing of various renally excreted drugs.(4, 5) Many different equations based on the available endogenous markers, most commonly creatinine and cystatin C, have been published over the years for children. **Chapter one** provides a table where the more recent equations are summarized. The abundance of equations using the same markers reflects differences in measurement techniques, in mathematical modeling and in patient populations. This impairs accuracy in clinical practice as has been shown in validation studies.(6)

The inaccuracy of GFR estimation can be overcome using gold standard clearance studies. With these methods GFR is calculated following intravenous injection of an exogenous marker followed by serial blood (and in some protocols urine) sampling. (7, 8) However these methods are invasive and time consuming and hardly suitable for daily practice. Also, there is considerable variation between the different clearance markers and protocols questioning the claim of being the "gold standard" for some of these methods. (9) Furthermore considerable variability in GFR over time makes a gold standard measurement a "snapshot" while estimated GFR from endogenous markers may provide a more stable assessment of kidney function, which could arguably be more clinically relevant.(10)

Therefore, for daily practice, endogenous serum markers and estimated GFR form the basis for clinical decision making and patients benefit most from increased accuracy of these methods

Single marker equations

When addressing the issue of (in)accuracy of GFR estimation from serum levels of endogenous markers, it is important to identify extra-renal factors affecting production or elimination of these markers. This will give insight into which patient groups are at risk for inaccurate GFR estimation by a specific marker, or which factors should be taken into account when developing an eGFR equation. Many of these factors have already been described in the literature. **Chapter one** provides an extensive overview of known confounding factors.(11) The most relevant factor in clinical practice is muscle mass

which is the primary determinant for the rate of creatinine production.(12) As muscle mass in children is correlated to height, height is incorporated in nearly all pediatric eGFR equations.(13-16) Other factors known to influence creatinine independent of glomerular filtration are the dietary intake of creatine and creatinine (17, 18) as well as tubular and intestinal secretion (19, 20) and are more difficult to adjust for in equations. These factors - besides constitutional variations in muscle mass between individuals of the same height - contribute to the inaccuracy in GFR estimation using serum creatinine and explain the added value of alternative eGFR markers.

Knowledge of these factors is needed to develop strategies based on patient characteristics that selectively use alternative markers, which are not affected by the same interfering factors. As an example, both cystatin C and the other two low molecular weight protein GFR markers beta-2 microglobulin and beta-trace protein are all affected by glucocorticosteroid therapy in a dose-dependent manner. (21) Here, **chapter two** of this thesis shows that creatinine is far less affected by glucocorticosteroid use. (22) Besides known confounding factors, which can potentially be adjusted for (23) there are unknown factors accounting for inter- and intra-individual variations in serum levels. Also, intra- and interassay variability as well as calibration differences contribute to bias and inaccuracy despite international efforts to standardize the creatinine and cystatin C assays markers. (24, 25) These determinants of inaccuracy of GFR estimation are illustrated in figure 7 of **chapter one.**

Combining markers: More is better?

In order to address this question, it is important to establish what constitutes “better” in the context of GFR estimation. Mostly eGFR equations are validated by comparison with measured GFR using a gold standard technique in terms of bias (systematic error), precision (random error) and accuracy.(26-28) Accuracy is a measure combining systematic and random error. It can be presented as the absolute difference between estimated and measured GFR or, more commonly, as the percentage of cases in which estimated GFR is within x percent of the measured GFR; the P_x accuracy. A P_{30} accuracy rate of 80% therefore means that for a patient with an actual GFR of 100 ml/min/1.73m^2 there is a 20% chance that the estimated GFR will be below 70 or above 130 ml/min/1.73m^2. (29) Most publications regard a p30 accuracy above 80% acceptable.(9, 30) Additionally some studies use the percentage of cases that correctly identify the CKD stage according to the KDOQI guidelines-as outcome measure.(31)

It is important to address the limitations to these widely used parameters for eGFR quality. First, there is difficulty in comparing results as slightly varying definitions are

used in different studies: mean versus median bias, (27, 32) absolute versus percentage bias (27, 33) or P_{10} versus P_{15} accuracy rates.(28, 34) Second, it is not uncommon to have both higher bias and higher accuracy rates or higher P_{30} but lower P_{10} accuracy rates for one equation compared to another, in which case it is difficult to state which equation is better. Third, the assumption that exogenous marker clearance studies measure true GFR is debatable. (9) In lieu of a true gold standard method more appropriate quality tests would be agreement tests, comparing new equations to existing non-perfect ones, such as Bland-Altmann limits of agreement tests.(35, 36)

Despite these limitations, it has been well established that combining two markers, either within complex equations (27, 37, 38), or by using the mean of two separate equations (6, 39) increases accuracy and decreases bias of the resulting GFR estimate. This can be explained by differences in marker characteristics, whereby the effect of confounding factors affecting one of the markers is partially compensated by use of another marker. In theory, adding more markers will increase accuracy further. However, **chapter three** shows that while combining any two out of the three markers creatinine, cystatin C and beta-trace protein clearly increases accuracy and diminishes bias compared to the respective single marker estimates, adding a third marker yields little to no improvement.(40) When it comes to accurate GFR estimation using endogenous markers, more is not necessarily better. This may reflect some overlap in physiology between the markers tested such as similarities in size, distribution across the body and rates of production. Also, as stated above, variability of the gold standard measurement as well as variability of true GFR over time has to be taken into account, which will not be corrected by using additional endogenous GFR markers. Therefore, as outlined in **chapter one**, accuracy of any eGFR equation will never reach 100%.

Combining markers: Smarter is better?

Instead of simply increasing the number of markers used to estimate GFR, accuracy can be better increased by personalizing equations based on patient characteristics. An important observation in this regard is the inverse relationship between the accuracy of GFR estimation and the difference between eGFR based on creatinine and cystatin C. This is an important finding in both **chapters five and six** (41, 42) and is consistent with previous reports in adults. (43) Although complex multi-marker equations perform slightly better, (27) calculating and comparing two separate simple eGFR equations allows the clinician to assess the level of accuracy of the eGFR by calculating the difference between eGFR based on creatinine and cystatin C, which has several advantages. First, in the vast majority of patients studied, there is little difference between the two GFR estimates which indicates high accuracy, even comparable with the variation observed between exogenous marker

studies.(9) In these cases, the mean of both eGFR estimates has sufficient accuracy for clinical decision-making. Second, in patients where a high difference between eGFR from the different markers is noted, this may be a clue to an underlying condition and thus have additional diagnostic value. This is illustrated by a case report where recirculation of creatinine from intraabdominal urinary leakage led to falsely elevated creatinine values while cystatin C remained normal.(44) Other factors causing discrepancies between markers such as muscle wasting and thyroid disbalance might similarly become apparent. (45) Additionally, in adults a markedly higher eGFR based on creatinine compared to cystatin C has been suggested to reflect differences in size selectivity of the glomerular filtration barrier and be associated with increased cardiovascular and all-cause mortality. Grubb et al have coined the term Shrunken Pore Syndrome for this phenomenon. (46) **Chapter four** of this thesis explores this issue in children.

Grubb et al proposed a stepwise approach to address differences between eGFRcys and eGFRcreat, the so-called Lund approach (47): If the difference between both methods is less than 40% the mean eGFR is reported while in cases of a larger discrepancy this is reported to the treating physician. The physician will then search for a known interference with either marker and select the marker, which is not affected for GFR estimation while the other marker is not used for GFR estimation. If no explanation is found and the discrepancy has been confirmed by a second measurement to exclude a laboratory error, a gold standard GFR measurement is considered. This approach has not received much attention and has not been validated by other centers. In our own hands, this approach led to lower accuracy than simply using the mean of two eGFRs (unpublished). The probable cause for this is found in **chapter six,** where - except for patients with spina bifida - the mean of eGFRcys and eGFRcreat outperforms either of the single marker equations. This suggests that the effect of glucocorticoid use for example is rather small compared to multiple other – yet unknown – covariates affecting creatinine and cystatin C production and metabolism. Using weighted means, i.e. entering different levels of α (0-1) for the equation $\alpha * eGFR_{crea} + (1- \alpha) * eGFR_{cys}$, based on patient characteristics, may further increase accuracy.

Clinical implications of this thesis

One of the main findings in this thesis is that using smarter combinations of two existing eGFR markers is more efficient than simply adding more markers. A practical suggestion for such a smarter combination is the modified Lund approach strategy presented in **chapter six**. A disadvantage of such a strategy is that it requires more calculations and reasoning than simply using a single or multi-marker equation. Therefore, implementation on the ward may be challenging – in particular if nephrologists are not involved.

Chapters five and six focus on height-independent equations. Most creatinine-based eGFR equations for children rely on height as a surrogate for muscle mass. This forms an obstacle to automatic eGFR reporting for children by laboratories, which is common practice for adults. Patient height is not generally available for reporting laboratories, in particular outside the hospital. Using height-independent eGFR equations allows for automatic reporting of eGFR. Simply entering serum levels of creatinine and cystatin C along with date of birth allows for automatic calculation of two GFR estimates, as well as the mean and the level of difference between the two. Laboratories can thus either report an accurate eGFR as the mean of a height independent creatinine and cystatin C based equation if the difference between the two is less than 40%, or the two separate eGFRs along with the level of their discrepancy if it exceeds 40%. In the latter case, the clinician can opt to use a weighted mean, based on patient characteristics, such as diagnosis or steroid use or to perform a gold standard measurement using an exogenous marker. The latter is becoming an increasingly viable option in selected patients due to the development of more simple clearance study protocols using new exogenous markers (48) or less invasive laboratory techniques. (49)

Another topic with possible profound clinical implications is the discussion around Shrunken Pore Syndrome, which is addressed in **chapter 4.** A large amount of data exists linking cystatin C and beta-trace protein to mortality and morbidity independently from GFR or creatinine.(50-54) The hypothesis of Shrunken Pore Syndrome is that this is caused by differences in size selectivity of the glomerular barrier, causing larger molecules such as cystatin C and beta-trace protein along with pro-inflammatory factors to accumulate. (46, 55, 56) Although we did find a correlation between cystatin C and beta-trace protein which was independent from GFR and creatinine, we could not draw definitive conclusions as we lack data on mortality in children. We suggest an alternative definition of Shrunken Pore Syndrome, which relies on a gold standard GFR measurement instead of a creatinine-based estimation. Our findings along with the mortality data from adult studies highlight the necessity of combining markers, specifically creatinine and cystatin C, as their prognostic value exceed the simple estimation of GFR.

Limitations of this thesis

One of the limitations of this thesis is the potential for selection bias in the patients studied. The entire thesis is based on a single database accumulated over a decade in pediatric nephrology patients who underwent an inulin clearance study on clinical grounds. This caused inclusion of patients with more severe morbidity and a slightly older age than typically found in general pediatric patient groups. This also precluded validation of the height-independent Pottel equation (57) in children under two years of

age. Our dataset has recently been included in a large pan-European registry "European initiative to optimize measurement and estimation of GFR" involving adult and pediatric nephrologists, epidemiologists and clinical chemists from all over Europe, with the aim of validating different eGFR equations using a total of more than 6600 clearance studies with simultaneous serum creatinine and/or cystatin C measurements. A subanalysis by centers showed that the Amsterdam cohort performed comparably to the other centers.(30)

Another limitation with respect to clinical implementation of the modified Lund approach with weighted means is the limited number diagnoses for which a weighted mean is reported. Also, the size of the diagnostic subgroups is rather small reducing the power to define exact weighting coefficients. It is likely that transplant patients, patients with neuromuscular disease, patients with eating disorders to name a few would also benefit from weighted means. A larger study population with more diagnostic groups could fill this knowledge gap.

Future developments

As stated above the next step will be expansion the weighted means approach to more patient groups with different potential confounding factors. Furthermore external validation of the strategy is needed in different study populations. This requires large patient numbers, which, due to the invasive nature of gold standard measurements can only be obtained by international collaboration. The "European initiative to optimize measurement and estimation of GFR" is an ideal platform for such a follow-up project as it provides data with a large diversity in patient groups, exogenous marker clearance study methods and levels of kidney function and has already produced valuable results.(30, 58) Another issue that needs to be addressed is discontinuity in GFR estimation at the transition from pediatric (below 18 years) to adult care. At present, most equations have been developed for use in either pediatric or adult patients. This is a problem for adolescent patients for whom the pediatric and adult eGFR equations often yield different results falsely suggesting a change in kidney function.(58) This problem can be overcome by using equations for the full age spectrum, such as FASage and FAScys for creatinine and cystatin C.(57, 59). In this context, **Chapter three** of this thesis can be regarded as the pediatric end of a full age spectrum equation for beta-trace protein. A collaboration with groups studying beta-trace protein in adults is underway and is expected to generate data to develop a full age spectrum equation for beta-trace protein.

Although not addressed in this thesis, little data exist regarding GFR estimation in premature babies and neonates, a vulnerable patient group with a high risk of kidney injury. A reason for this is the invasive nature of conventional clearance studies with exogenous markers,

which is essential for the validation of existing and development of novel GFR estimating equations. The novel technique of iohexol measurement in dried capillary blood samples gives the opportunity to perform single injection iohexol clearances in young children with minimal patient inconvenience.(49, 60, 61)

In the coming years new potential markers are likely to be studied. Recently the peptide proenkephalin has been linked to GFR.(62, 63) Also, a metabolomics approach, using mass-spectrometry to identify potential endogenous markers of GFR has yielded promising results. (64-67) As all endogenous markers are likely to have non-GFR related factors influencing their serum levels it is rather unlikely that any single new marker will vastly increase accuracy. However, combining old and new endogenous markers may still yield some improvement in GFR prediction.

Chapter four discusses a possible link between serum levels of cystatin C and beta-trace protein independently from creatinine, which could be explained Shrunken Pore Syndrome in children. This link can be further examined by expanding a recent study in adults in which proteomics panels were used to compare serum levels of many atherosclerosis promoting factors between patients with and without Shrunken Pore Syndrome to our pediatric population.(56) While this link along with its clinical implications has been amply been debated, (55, 68) a clear histological substrate is missing. More light can be shed on this recently proposed disease by either electron microscopy of glomeruli from biopsies in patients where clinically shrunken pore syndrome is apparent, or by measuring the elimination rates of different exogenous markers with different molecular sizes, to rule out similarities in synthesis between the endogenous markers.

General conclusion

Increased accuracy of eGFR can be achieved using smart combinations of existing markers, in particular creatinine and cystatin C.

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Summary

Glomerular filtration rate (GFR) is an important indicator of kidney function and accurate knowledge of a patient's GFR is necessary for the recognition and follow-up of renal disease as well as for dosing of renally excreted medication. GFR can be measured fairly accurately by intravenous injection of an exogenous marker and calculated either from the rate of decline of serum levels or the rate at which the marker appears in the urine. These measurements are invasive, time consuming and costly and therefore seldom performed in a clinical setting. Usually GFR is estimated using serum levels of endogenous markers.

Chapter one starts by describing the requirements of an endogenous marker. Subsequently currently known endogenous markers for kidney function, i.e. creatinine, urea, cystatin C, beta-trace protein and beta-2 microglobulin are discussed in terms of physiology, analytical methods and specifically their use in children. Different strategies for calculating estimated GFR from serum levels of the endogenous markers are outlined along with a table summarizing eGFR equations for children published in recent years.

While the influence of corticosteroids on serum levels of cystatin C has been well studied, little is known about the effects of these drugs on the most widely used marker for eGFR, creatinine. **Chapter 2** is a retrospective study using both longitudinal and cross-sectional comparisons of the bias of creatinine based eGFR with and without corticosteroid use. No significant effect of corticosteroid use was found in our population and while the longitudinal analysis showed a trend towards underestimation of GFR in the corticosteroid group, the cross-sectional analysis trended towards dose-dependent overestimation of GFR with steroids, strengthening the conclusion that corticosteroids have no effect on serum creatinine independent from changes in GFR.

Beta-trace protein is a relatively new marker of kidney function and only a few beta-trace protein-based eGFR equations exist for children. Recently, a new method was developed to create creatinine- and cystatin C-based equations for the full age spectrum (FAS) using rescaled serum levels based on normal values found in healthy populations. In **Chapter 3** we extend this approach to create a new beta-trace protein based eGFR equation for a pediatric population, which we compare to the creatinine- and cystatin C-based FAS equations. Our new equation is slightly less accurate than the cystatin C based equation in the general population. However, in specific populations such as patients with malignancy the beta-trace protein equation out performs the other two. Combining any two equations improves accuracy, however combining all three does not further improve performance.
Recently a condition termed "Shrunken Pore Syndrome" (SPS) has been proposed

to explain the link between increased mortality and cystatin C independent of kidney function in adults. According to this theory smaller glomerular pore size leads to retention of cystatin C, along with pro-inflammatory factors, while the smaller creatinine molecule is still freely excreted. **Chapter 4** explores this phenomenon in children by comparing discrepant eGFR results between creatinine- and cystatin C-based equations with beta-trace protein-based eGFR, as the latter is similar in size to cystatin C. The results show a link between cystatin C and beta-trace protein which is independent from creatinine and measured GFR, suggesting that SPS does exist in children, too. We also propose an alternative definition of SPS using gold standard measurement of GFR to eliminate abnormally low creatinine production in the definition of SPS.

Most pediatric eGFR equations based on creatinine require knowledge of patient height, which makes it difficult for laboratories to directly report eGFR as is common practice for adults. As described in chapter 3, studies have shown improved accuracy when creatinine- and cystatin C-based equations are combined. **Chapter 5** shows that highly accurate eGFR can be reported by using the mean of the height-independent FASage and FAScys equations. This allows for direct eGFR reporting by laboratories as opposed to serum concentrations of the markers.

Building on the previous chapter, **chapter 6** explores ways to further increase the accuracy of eGFR estimation using the mean of a creatinine- and cystatin C-based equation. One approach is to use the geometric mean i.e. the square root of the product between creatinine- and cystatin C-based eGFR, which showed slightly higher accuracy rates than the arithmetic mean, i.e. one-half of the sum of the creatinine- and cystatin C-based eGFR. We explored if adjusting the weight of the creatinine- and cystatin C-based equation within the arithmetic or geometric mean improves accuracy. In our general population equal contributions of creatinine and cystatin C are most accurate. However specific groups, such as patients with malignancy, nephritis, spina bifida had higher accuracy rates with different relative contributions of creatinine and cystatin C. Finally, accuracy rates of the means are very high in patients with a low level of discrepancy between the creatinine- and cystatin C-based eGFR. These findings prompt the proposition of a strategy for accurate eGFR reporting. If the discrepancy between height-independent eGFR based on creatinine and cystatin C is less than 40%, which was the case in 83% of our population, the mean between the two should be reported and has an accuracy of 93%. If the discrepancy exceeds 40%, identify a patient characteristic, such as diagnosis of malignancy of spina bifida, that calls for a weighted mean and use the appropriately weighted mean. If no such patient characteristic is apparent, repeat the measurement to exclude a measurement error and consider performing a gold standard measurement using an exogenous marker.

Curriculum vitae

Emil den Bakker was born on June 3rd 1989 in Jerusalem. He grew up in Almere, the Netherlands, where he attended het Baken Park Lyceum high school, from which he graduated in 2007. He then studied medicine at the Vrije Universiteit in Amsterdam, during which time he worked on a research project about maternal and perinatal audits in Ifakara, Tanzania. This resulted in his first peer-reviewed scientific publication. In 2013 he returned to Tanzania for an extra-curricular pediatric internship at Haydom Lutheran Hospital. After graduating in 2014, he started working on a research project about markers for kidney function. At the same time he worked as a resident (ANIOS) at the pediatric department of het Diakonessenhuis in Utrecht for a year, after which he started at het Flevoziekenhuis, again as resident in the pediatric department. While working here he decided to try to expand the research project, which initially was aimed at an congress abstract, to a full PhD program. Meanwhile he continued clinical work in het Flevoziekenhuis and in 2018 at het Wilhelmina Kinderziekenhuis in Utrecht. In September 2018 he got accepted into the Pediatric residency training program at the Amsterdam UMC, location VUMC. Since January 2019 he has been working in het OLVG Oost in Amsterdam as a part of that program.

Emil is living in Weesp together with Yolanda Wallenburg. They are expecting their first child.

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